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Experimental approaches and clinical opportunities in metabolic liver diseases

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EXPERIMENTAL APPROACHES AND CLINICAL OPPORTUNITIES IN METABOLIC LIVER DISEASES

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Cover art illustration: Artistic rendition of immunofluorescence labeled hepatocytes showing DNA replication, seen through a liver outline, by Ahmad Karadagi.

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THESIS FOR DOCTORAL DEGREE (Ph.D.)

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“If you thought that science was certain – well, that is just an error on your part.”
― Richard P. Feynman

POPULAR SCIENCE SUMMARY OF THE THESIS

The liver is an organ responsible for many functions such as detoxification of toxins ingested or created by the body. It is also responsible for the production of numerous molecules important for life and function. Inherited genetic mutations give rise to a plethora of different disease of these metabolic liver functions. These inherited metabolic liver diseases usually present in infancy or at an early age. There are also genetic/metabolic liver diseases where the symptoms do not appear until adulthood. Symptoms can arise from the liver itself or elsewhere in the body depending on the missing liver-made product and function, such as developmental inhibition of the brain, uncontrolled bleeding or lung disease.

Many metabolic liver diseases can be treated—even cured by liver transplantation or kept at bay by medicine or strict dietary restrictions. In this thesis, firstly, a possible liver protective aspect of replacement therapy for the liver disease, alpha 1-antitrypsin deficiency was studied. Secondly, alpha 1-antitrypsin deficiency was used as a model to explore new way of using modified mRNA to treat metabolic liver disease. Lastly, the consequences of reusing livers from metabolic liver disease patients to transplant another patient with liver disease was studied.

In the first study (**Study I**), the liver disease alpha 1-antitrypsin deficiency was examined, a liver disease where a mutation and a faulty protein causes death of liver cells leading to cirrhosis and increased risk of hepatocellular cancer. The deficiency also causes increased risk for premature chronic obstructive lung disease when not enough alpha 1-antitrypsin is present to protect the lungs from damaging enzymes secreted by white blood cells. We tested whether giving purified alpha 1-antitrypsin can reduce the amount of the mutant form of the protein and thus actually reduce liver damage in addition to the lung protection. Our results from liver cells isolated from surgically removed human livers, blood samples and lung tissue indicated a possibility to reduce liver damage caused by the faulty protein when treating with refined alpha 1-antitrypsin. These finding are of importance as treatment with refined alpha 1-antitrypsin is not currently available for alpha 1-antitrypsin deficient patients in Sweden.

In the second study (**Study II**), we tested restoring functional alpha 1-antitrypsin by giving laboratory made messenger RNA, which is the formula used by the body to make proteins. We performed studies in human liver cells and mice, in which we were able to deliver the messenger RNA into the liver cells and were subsequently able to detect the protein product, which was functional. Taken together, our results showed promise in delivering and restoring protein levels in alpha 1-antitrypsindeficiency and in extension this may also be promising in other diseases.

Study III is a clinical study evaluating the impact of so-called domino liver transplantation. The procedure where the removed liver from a metabolic liver disease patient undergoing liver transplantation is used to treat another patient. In Sweden, mainly explanted livers from patients with familial amyloidotic polyneuropathy (FAP), locally known as “Skelleftesjukan”, is used as donor livers for domino liver transplantation. Examining patient

records from 2007–2017 showed that this domino transplant program was able to be conducted without affecting the waiting time or survival for the included patients or standard patients.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Levern är ett organ med en rad livsviktiga funktioner, såsom avgiftning av toxiner som skapas i kroppen genom olika processer eller som intages. Levern ansvarar också för produktionen av de flesta proteiner och molekyler som cirkulerar i blodet och dessa har viktiga funktioner i kroppen. Därför kan nedärvda genetiska mutationer i levern ge upphov till en uppsjö av olika sjukdomar. Vanligtvis framträder ärftliga metabola leversjukdomar i spädbarnsåldern eller tidig barndom. Dock förekommer det sjukdomar med symtom som uppträder först i vuxen ålder. Symtom från metabola leversjukdomar kan uppstå i levern, eller andra organ som är beroende av leverns funktioner eller produkter. Exempelvis; mental utvecklingshämning i hjärnan, okontrollerad blödningsbenägenhet eller lungsjukdom. Metabola leversjukdomar kan behandlas och botas genom levertransplantation. Symtomen kan ibland också lindras med mediciner eller stränga kostrestriktioner.

Inom ramen för denna avhandlingen har en möjlig leverskyddande mekanism av substitutionsbehandling med alfa 1-antitrypsin studerats. Därtill användes alfa 1-antitrypsinbrist som sjukdomsmodell för att prova den nya metoden att använda meddelande RNA för att behandla metabola leversjukdomar. Slutligen, studerades även effekterna av att använda levern från patienter med metabola leversjukdomar för att möjliggöra transplantation av ytterligare en annan individ.

I den första studien (**Study I**) undersökte vi sjukdomen alfa 1-antitrypsinbrist, en leversjukdom där en mutation och ett felaktigt protein kan orsaka både leverskada och lungsjukdom. Vi utforskade effekterna på genuttrycket av den gen som kodar alfa 1-antitrypsin i bl.a. leverceller, lungvävnad samt blod efter substitutionsbehandling med alfa 1-antitrypsinprotein framrenat från human plasma. Våra experimentella resultat tyder på att genuttrycket (produktionen) av alfa 1-antitrypsin minskar vid tillförsel av samma protein. Således talade data för att en skyddande effekt på leverceller kan uppstå då produktionen av det muterade och skadande protein minskar när substitutionsterapi med renat alfa 1-antitrypsin ges. I Sverige ingår inte substitutionsbehandling med renat alfa 1-antitrypsin i behandlingsprogrammet för alfa 1-antitrypsinbrist patienter.

I den andra studien (**Study II**) testade vi att återställa tillräcklig mängd friskt och funktionellt alfa 1-antitrypsin genom att administrera konstgjort s.k. meddelande RNA, alltså den mall som används av kroppen för att tillverka proteiner. Vi utförde försök på mänskliga leverceller och på möss. Vi fann att det var möjligt att leverera meddelande-RNA till levercellerna och därefter kunde vi detektera den tillverkade och fullt fungerande proteinprodukten. Sammanlagt visade resultatet på lovande möjligheter att återställa proteinnivåer vid alfa 1-antitrypsinbrist och metoden kan i förlängning tillämpas även på andra sjukdomar.

I den slutliga studien **(Study III**) fokuserade vi på den kliniska aspekten av metabola leversjukdomar och levertransplantation. Vi undersökte så kallad domino-levertransplantation, en operation där den borttagna levern från en patient med metabol

leversjukdom (främst familjär amyloidos med polyneuropati, även kallad ”Skelleftesjukan”) som genomgår levertransplantation återanvänds för att behandla en andra patient. Undersökning av patientjournaler från 2007–2017 visade att domino-transplantationer kunde genomföras utan att det påverkade väntetiden eller överlevnaden för dem inkluderade patienterna eller levertransplantationer av övriga patienter.

ABSTRACT

Metabolic liver diseases are uncommon individually, however, collectively they represent significant cause of morbidity and mortality globally. The liver has many diverse functions, such as; detoxification from hazardous compounds, either ingested or produced by the body, and production of vital proteins, e.g. coagulation factors and acute phase proteins. Liver disease manifestations are therefore immensely diverse and may present both in a strictly hepatic or extra-hepatic manner. Metabolic liver diseases are usually monogenic, meaning a single point mutation is causing the propagation of the disease. Orthotopic liver transplantation has been employed for the treatment of a broad range of metabolic liver diseases and is indeed a curative method in most instances.

In this thesis, we focused on alpha 1-antitrypsin deficiency–one of the monogenetic liver diseases with both hepatic and pulmonary manifestations. In **Study I**, we examined and showed that alpha 1-antitrypsin replacement therapy may also have an impact on hepatocytes and the propagation of liver disease. Replacement therapy is currently only reserved for pulmonary manifestation of alpha 1-antitrypsin deficiency. Addition of exogenous alpha 1-antitrypsin protein reduced alpha 1-antitrypsin (SERPINA1) mRNA expression in primary human hepatocytes from both deficient and proficient patients. Furthermore, similar results were seen in lung tissue samples from deficient patients. Altogether, the results may indicate a feedback mechanism where adequate circulating alpha 1-antitrypsin causes a reduction in its own production and replacement therapy may be beneficial for the reduction of hepatic damage caused by mutant alpha 1-antitrypsin.

Study II focused on investigating the novel promising mRNA-based therapies for protein replacement in alpha 1-antitrypsin deficiency. Modified mRNA encoding alpha 1-antitrypsin protein was delivered *in vitro* to primary human hepatocytes from both deficient and proficient patients. Subsequently *in vivo* delivery of modified mRNA was conducted in wild type mice and the NSG-PiZ AATd mouse model. Translated and functional AAT protein was detected both *in vitro* and *in vivo*.

At time of liver transplantation, explanted livers from metabolic liver disease patients may be recovered and used as a graft for another patient with chronic liver disease in a procedure called domino liver transplantation. In **Study III**, we evaluated the domino liver transplant program at Karolinska University Hospital, Huddinge from 2007–2017. Patients undergoing domino liver transplant had similar survival rate and time spent on waiting list as age and diagnosis matched patients undergoing deceased donor liver transplantation.

To conclude, we have shown hepatic benefits of replacement therapy in alpha 1-antitrypsin deficiency and explored mRNA therapy as a novel way to replace the missing protein. We have further evaluated the domino liver transplant program, in which livers from metabolic liver disease patients are used as donor grafts and concluded that the program has enabled additional transplantations without affecting patients' waiting time or survival.

LIST OF SCIENTIFIC PAPERS

I. **Karadagi A**, Johansson H, Zemack H, Salipalli S, Mork LM, Kannisto K, Jorns C, Gramignoli R, Strom S, Stokkeland K, Ericzon BG, Jonigk D, Janciauskiene S, Nowak G, Ellis ECS. Exogenous alpha 1-antitrypsin down-regulates SERPINA1 expression.
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Submitted to Am J Transplant

ADDITIONAL PUBLICATIONS NOT INCLUDED IN THESIS

I. Jorns C, Nowak G, Nemeth A, Zemack H, Mork LM, Johansson H, Gramignoli R, Watanabe M, **Karadagi A**, Alheim M, Hauzenberger D, van Dijk R, Bosma PJ, Ebbesen F, Szakos A, Fischler B, Strom S, Ellis E, Ericzon BG. De Novo Donor-Specific HLA Antibody Formation in Two Patients With Crigler-Najjar Syndrome Type I Following Human Hepatocyte Transplantation With Partial Hepatectomy Preconditioning.
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V. Tokodai K, Kumagai-Braesch M, **Karadagi A**, Johansson H, Agren N, Jorns C, Ericzon BG, Ellis E. Blood Group Antigen Expression in Isolated Human Liver Cells in Preparation for Implementing Clinical ABO-Incompatible Hepatocyte Transplantation.
J Clin Exp Hepatol. 2020 Mar-Apr;10(2):106-13.

CONTENTS

LIST OF ABBREVIATIONS

AATd	Alpha 1-antitrypsin deficiency
AAT	Alpha 1-antitrypsin
Pi	Protease inhibitor
FAH	Fumarylacetoacetate hydrolase
NTBC	2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione
PKU	Phenylketonuria
PAH	Phenylalanine hydroxylase
MSUD	Maple syrup urine disease
BCKDH	Branched-chain-alpha-keto acid dehydrogenase complex
UGT1A1	Uridine diphosphate glucuronosyltransferase 1A1
CRISPR	Clustered regularly interspaced short palindromic repeats
Cas9	CRISPR associated protein 9
TTR	Transthyretin
LT	Liver transplantation
DLT	Domino liver transplantation
FAP	Familial amyloidotic polyneuropathy
FAPWTR	FAP world transplant registry
TLR	Toll like receptor
OSM	Oncostatin M
PBMC	Peripheral blood mononuclear cells
LPS	Lipopolysaccharide
WB	Western blot
ELISA	Enzyme-linked immunosorbent assay
HBSS	Hank’s balanced salt solution
EGTA	Ethylene glycol tetra acetic acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
PCR	Polymerase chain reaction
SNP	Single nucleotide polymorphism
eGFP	Enhanced green fluorescent protein
RIPA	Radioimmunoprecipitation assay buffer

AST	Aspartate aminotransferase
ALT	Alanine aminotransferase
ALP	Alkaline phosphatase
CK	Creatine kinase
GGT	Gamma-glutamyl transferase
DAB	3,3’-diaminobenzidine
Ppib	peptidylprolyl isomerase B (Mus musculus)
DapB	dihydrodipicolinate reductase
CRC	Colorectal cancer
HCC	Hepatocellular carcinoma
PFIC2	Progressive familial intrahepatic cholestasis type 2
nonDLT	Non-Domino liver transplantation
convLT	Conventional liver transplantation
HR	Hazard ratio
CI	Confidence interval

1 INTRODUCTION

1.1 INHERITED GENETIC/METABOLIC LIVER DISEASES

Given the broad range of hepatic functions, inherited liver diseases present in a wide spectrum of clinical symptoms and manifestations. Monogenic disorders are a group of diseases caused by a defect in a single gene causing a manifest disease. Alpha 1-antitrypsin deficiency (AATd) and Wilson's disease are examples of the more common genetic diseases of the liver. However, many more exist and are usually coupled to a point mutation affecting proteins involved in the physiological metabolism, of which a handful will be addressed in this thesis. Protein mutations can either lead to a loss of function, a deficiency or in other cases to a gain of unwanted function resulting from the mutation. A mutation may for example lead to a defective protein that is degraded or is unable to carry out its function, leading to a complete loss of function. In other cases, genetic mutations may lead to partial loss of function and a deficiency of the target protein, where although a mutation is present, some remaining function is still available. These deficiencies may present milder symptoms or be sufficiently corrected by available therapies. In other cases, the mutations introduce structural changes in the proteins leading not only to dysfunction but also to aggregation which in turn leads to cytotoxic qualities, resulting in a gain of unwanted function.

The disease manifestation and symptoms may be hepatic, mainly affecting the liver, or present in an extrahepatic manner with symptoms mainly from other organs. Many carriers of mutations may never develop symptoms or develop mild forms of the disease, never requiring intervention. These genetic metabolic liver diseases are indeed exceedingly heterogeneous. Even though mutations in hepatic proteins and function is the common origin for these liver diseases, the clinical presentation and consequently the therapeutic approaches may vary greatly without a single all-encompassing curative strategy.

One can argue that liver transplantation is a logical therapy for metabolic liver diseases. Orthotopic liver transplantation is indeed a curative treatment, nevertheless associated with the burden and risk of life-long immunosuppression and other transplant-related complications. Moreover, whole organ transplantation is severely restricted by available organs for transplantation, which limits the number of patients that can be transplanted. Additionally, surgical and physical aspects may hinder transplantations, where matching the correct size of the organ is crucial, especially in newborns. These aforementioned factors may therefore hamper or halt whole liver transplantations to the extent needed. Yet, inherited metabolic diseases are rare but still represent a significant group of patients undergoing liver transplantation.

An excessive capacity often exists in these metabolic pathways and a small amount of reserved function or protein production may be sufficient to correct the disease phenotype. This is the premise for hepatocyte transplantation, in which a relatively small number of hepatocytes are transplanted instead of a whole organ.

Understandably, alternative therapies to liver transplantation for metabolic/genetic liver diseases are needed. This thesis explores a number of genetic and metabolic liver diseases that are being treated by liver transplantation today and focuses on AATd. Genetic liver diseases are well suited as targets for novel biomolecular treatment strategies, including gene modification/replacement or modified mRNA therapy, as was studied in this thesis.

1.1.1 Alpha 1-antitrypsin deficiency

Alpha 1-antitrypsin deficiency is a genetic disease that can lead to an early onset (fourth or fifth decade of life) of chronic obstructive pulmonary disease, panniculitis, systemic vasculitis, inflammatory/autoimmune, neoplastic disease and liver disease (1). In 1963 Laurell and Eriksson first described the condition based on observations of electrophoresis of sera from patients in Sweden (2). Later, the link between AATd and liver disease was shown by Sharp et al. in 1969 (3).

In the healthy individual, alpha 1-antitrypsin (AAT) protein is an acute phase protein and mainly secreted by hepatocytes and is abundantly circulating in blood where it neutralizes proteases, specifically neutrophil elastase in the lungs. The protein is encoded by the protease inhibitor (Pi) locus, SERPINA1 gene on chromosome 14q32.1 (4). It is mainly produced by hepatocytes and is a water-soluble glycoprotein, with a molecular weight of 52 kDa. In AATd, a point mutation causes a mutant form of the protein to be produced, which aggregates in the liver causing hepatic damage leading to cirrhosis, hepatocellular carcinoma and more commonly pulmonary disease due to low levels of circulating protein (5). The pathological mechanisms are mediated by the point mutation resulting in an amino acid change that yields an unfavorably charged protein, leading to misfolding and protein aggregation. The aggregated protein accumulates and is trapped in the rough endoplasmic reticulum of affected hepatocytes. There are several variants of the AAT protein and they are classified depending on their migration speed on isoelectric pH gradient. The ones clinically relevant and common are medium (M), slow (S) and very slow (Z), and are named PiM for the healthy variant and PiZ (homozygous PiZZ) for the variant found in severely deficient patients (6). The Z allele is a single-nucleotide polymorphism caused by a point mutation causing the substitution of Glu342 with Lys (7). Other variants including very rare *null* mutations exist and have been reported, however, S and Z mutations remain the most clinically relevant variants (8, 9).

The disease manifestation is very diverse and encompasses both pulmonary and/or hepatic involvement ranging from modest symptoms to complete organ failure necessitating organ transplantation. Pulmonary damage is caused by a loss of function—a lack of circulating protein protecting lung tissue from the protease, elastase secreted by neutrophils as a defense against pathogens. This damage is driven by the formation of emphysema and these patients are highly susceptible to damage caused by smoking leading to the rapid development of chronic obstructive pulmonary disease at a young age. Hepatic involvement and pathology ranges from early neonatal cholestasis requiring prompt liver transplantation all the way to

first discovery of liver cirrhosis at 50 years of age. Acutely ill neonates only accounts for a small fraction while the majority of patients go on unnoticed and up to 15% develop cirrhosis and hepatocellular carcinoma (10). Not all PiZZ individuals develop liver disease, longitudinal studies of Swedish patients showed a 10% prevalence of liver disease among PiZZ individuals. Male gender, age over 50 years, elevated liver enzymes, hepatitis and diabetes mellitus were risk-factors (11). Previous studies by the same group indicated liver disease (cirrhosis or hepatocellular carcinoma) as cause of death among 20% of PiZZ individuals (12). AATd is underdiagnosed, the Swedish newborn screening study of Sveger in 1976 showed a prevalence of severe homozygous PiZZ in 1:1639 of newborns (13). Recent data may suggest a prevalence of 1:4368 in Sweden, and an estimation of 253 404 total PiZZ individual worldwide (14).

In the lungs there is a loss of function and in the liver the pathology is mediated by the gain of unwanted function, however—it is a toxic effect. In deficient patients the amount of circulating protein is less than 15% of normal levels, which are 1-2 mg/ml. Reaching less than 11 mmol is the lower limit for the development of lung-associated symptoms (15).

Today, plasma purified AAT replacement is the only medical therapy available for the treatment of AATd. Liver transplantation is employed in advanced cases of cirrhosis but is unavailable to all patients with severe deficiency due to organ shortage. Hepatocyte transplantation has been proposed as an alternative therapy. Augmentation therapy with AAT from pooled human serum is only indicated for adults with lung disease and is not available in Sweden (16-19). The hepatic aspect of augmentation therapy is not very well studied as patients with liver manifestations from AATd usually do not display lung disease and are therefore not considered for augmentation therapy. This piece of missing knowledge was addressed in (**Study I**). Therapies involving small molecules are being explored as an alternative way to decrease the morbidity associated with the disease. These therapies include molecular chaperones guiding the AAT protein and preventing its accumulation in the endoplasmic reticulum of hepatocytes (20), which unfortunately at least using phenylbutyrate, a promising compound in animal trials with PiZ mice has not proven very successful in humans (21).

Autophagy, the process by which cells remove unwanted and dysfunctional protein is another approach that is being investigated to reduce accumulated globules of dysfunctional AAT protein. Chemicals such as tamoxifen, fluphenazine, carbamazepine, and the immunosuppressant and mTOR inhibitor rapamycin have all demonstrated promising effects in increasing the autophagy process (22).

Another exciting approach is the use of genetic engineering and gene therapy. Using antisense oligonucleotide inhibitor to silence or “knock-down” the SERPINA1 gene has been demonstrated to show reduction of circulating AAT in non-human primates and also to the reduction of liver fibrosis in PiZ transgenic mice (23, 24). Further, it was demonstrated by Mueller et al. that a single intramuscular injection of recombinant adeno-associated virus

serotype 1 AAT vector was able to sustain protein production at 2% of target expression up to 5 years (25-27). Therapeutic genome editing for AATd has also been conducted using Clustered regularly interspaced short palindromic repeats / CRISPR associated protein 9 (CRISPR/Cas9) genome editing, showing promising results (28).

1.1.2 Familial amyloidotic polyneuropathy

Familial amyloidotic polyneuropathy is a hereditary, autosomal dominant, systemic amyloidosis disorder caused by a mutation in the gene encoding transthyretin (TTR). Reports of FAP first came from Portugal, Japan, and Sweden where the disease is endemic (29). According to the Swedish National Board of Health and Welfare, approximately 350 patients have FAP in Sweden. Many variants exist, however in Sweden the Val30Met variant, where methionine replaces valine is most common. This mutant variant changes the solubility of the protein causing precipitation and fibrillary amyloid deposits of the mutant protein in the extracellular space in the tissue. Amyloid deposits are mainly seen in the perineurium causing progressive autonomic and peripheral polyneuropathy (30, 31). Although 95% of this tetrameric protein is produced in the liver, the disease is strictly extrahepatic. Most commonly the amyloid deposits in kidneys, intestine or the heart (32-34). FAP symptoms include painful polyneuropathy, often involving lower extremities. Disease progression further involves other organs such as the heart and intestines. Historically, a mean survival of 9-13 years is reported once symptomatic disease is presented (35, 36). Considering the hepatic nature of this disease, a treatment option via liver transplantation was speculated. It was subsequently suggested by Holmgren et al. that liver transplantation indeed could halt the progress of the disease and improve the dismal outcome of the disease (37). Liver transplantation has now become standard care for FAP patients, with over 2000 liver transplantation performed for FAP worldwide according to FAPWTR (38).

1.1.3 Wilson's disease

Wilson's disease is a progressive genetic, autosomal recessive disease of copper metabolism. The disease was first described in 1912 by the neurologist SA Kinnier Wilson (39). It was later found to be propagated by mutations in the ATP7B gene. The ATP7B transporter is transmembrane protein responsible for the transport of copper for incorporation into ceruloplasmin for circulation to where it is needed in the body. The transporter is also needed for excretion of excess copper from hepatocytes into bile. The mutation leads to a loss of function of the ATP7B transporter, leading to copper accumulations in the hepatocytes causing damage or leaked into circulation where it may be deposited in other tissues including the brain. Symptoms are of chronic hepatitis and eventually liver cirrhosis with ascites, edema, coagulopathy, hepatic encephalopathy, portal hypertension and splenomegaly. Extrahepatic symptoms are neurological (tremor, dysarthria, dystonia and ataxia), psychiatric and liver disease. A pigmented ring of copper deposits around the iris called Kayser-Fleischer ring is present. Medical treatment includes copper chelators and zinc. With an incidence of 1/1000 000, 75 known patients are currently identified by the Swedish National Board of

Health and Welfare. Liver failure due to Wilson's disease is treated successfully with liver transplantation, which is also curative as functional ATP7B is restored (40).

1.1.4 Hereditary tyrosinemia type 1

The last step of the metabolism of the amino acid tyrosine is catalyzed by the enzyme fumarylacetoacetate hydrolase (FAH) and mutations causing a deficiency in FAH results in hereditary tyrosinemia type 1 (41). Loss of the function of FAH and impaired tyrosine metabolism leads to accumulation of fumarylacetoacetate which in turn causes mutagenic and cytotoxic insult to hepatocytes, causing caspase cascade induced apoptotic liver damage (42). Both acute and chronic manifestations may present with acute liver dysfunction leading to liver failure and liver cirrhosis with increased risk of hepatocellular carcinoma respectively in addition to renal damage and impaired intellectual development (43). Approximately one child is born with this mutation each year in Sweden. Treatment efforts are focused on decreasing tyrosine by dietary restriction. Also, administration of 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC), which inhibits the formation of maleylacetoacetate and fumarylacetoacetate is used (44). Liver transplantation replaces the deficient enzyme and is considered curative.

1.1.5 Glycogen storage disease type 1

Deficiency of the hepatic glucose-6-phosphatase or glucose-6-phosphate transporter causes impaired glycogenolysis and gluconeogenesis (45, 46). The deficiency is a loss of function and in turn causes diminished glucose homeostasis with associated hypoglycemia and increased lactate. The condition is also associated with alternative metabolic pathways leading to accumulation of glycogen in both liver and kidney. Further deranged metabolic consequences include hypercholesterolemia, hypertriglyceridemia and hyperuricemia. Some patients may develop adenomas of the liver. Dietary intervention to correct hypoglycemia is used and liver transplantation is recommended in unresponsive individual or those with hepatic adenomas (46).

1.1.6 Progressive familial intrahepatic cholestasis

Progressive familial intrahepatic cholestasis (PFIC) contains a group of heterogeneous autosomal recessive diseases of the liver and specifically bile formation and transport. It is a rare disease with incidence of 1-2/100 000 newborns. Three distinct mutations give rise to three types of PFIC. PFIC1 is caused by a mutation in the ATP8B1 gene encoding FIC1 protein, an ATP-dependent aminophospholipid transporter. PFIC2, is propagated by a mutation in the ABCB11 gene encoding the BSEP protein, a transmembrane ATP-dependent bile acid transporter. Finally, PFIC3 is caused by mutation in the ABCB4 gene encoding MDR3, an ATP-dependent phosphatidylcholine transporter. The disease is hallmarked by cholestasis as bile is not transported and not concentrated correctly due to non-functional transporters. Symptoms are of cholestasis, including jaundice and appears in the neonatal or early childhood time period. The disease usually progresses to liver fibrosis and end-stage

liver disease. Treatments include ursodeoxycholic acid, and biliary diversion in selected patients. Many patients undergo curative liver transplantation, however the disease is a candidate for genetic therapies (47).

1.1.7 Urea cycle disorders

Another common group of metabolic liver disease is defects in the urea cycle metabolism propagated by a loss of function in key enzymes. The urea cycle pathway is mainly in the liver and a pathway of clearing toxic ammonia produced by protein degradation and was first described by Krebs and Henseleit in 1932 (**Figure 1**). Defects cause diseases by mutation in any of the six enzymes in the urea cycle resulting in accumulation of toxic ammonia and subsequent irreversible neurological damage. The disease generally presents in the neonate period with poor feeding, fatigue and lethargy followed by neurological symptoms of autonomic dysfunction, loss of reflexes and seizures. Treatments include compounds that offer alternative pathways for nitrogen excretion, e.g. sodium benzoate or phenylbutyrate which are conjugated with glycine and glutamine respectively, thus removing nitrogen. Dialysis with hemofiltration may be needed acutely to control high ammonia levels. Otherwise, meticulous dietary planning and restrictions are need. Substitution of necessary amino acids is also required. The only curative treatment is liver transplantation with impact on patient's morbidity and mortality (48).

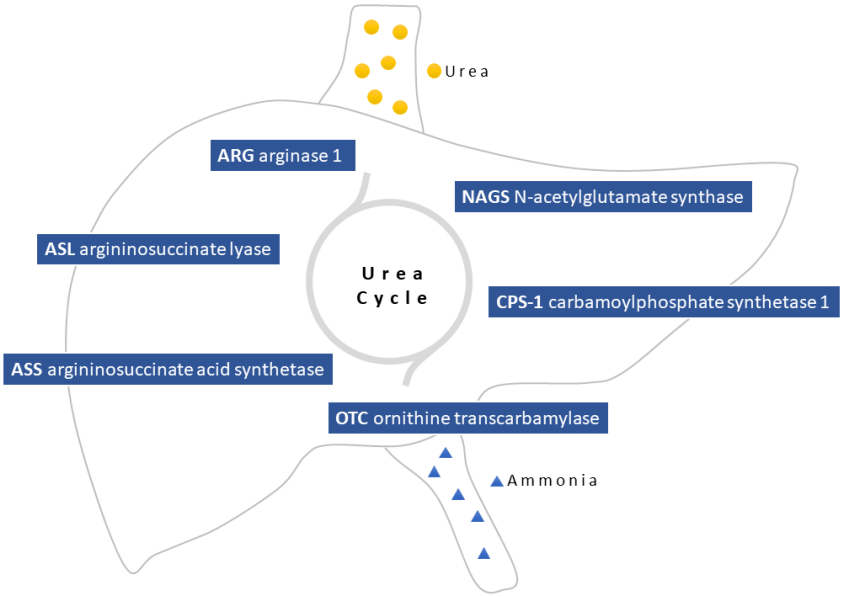

Figure 1. Enzymes of the urea cycle.

1.1.8 Phenylketonuria

One of the better-known metabolic diseases is phenylketonuria (PKU) caused by a mutation in the phenylalanine hydroxylase (PAH) enzyme gene causing accumulation of the amino acid phenylalanine, which in turn leads to seizures, intellectual disability, mental and behavioral disorders (49). PKU is more colloquially known due to the newborn screening

program for multiple inherited diseases common in many countries, in Sweden the program is referred to as the PKU-test. Although there is a lack of consensus regarding the threshold for therapeutic blood phenylalanine concentrations, the main therapeutic approach is to limit dietary phenylalanine and maintaining a low-protein diet. Usually, individual diets must be created with strict restriction of protein content. A subgroup of patients my benefit from treatment with sapropterin, which is a synthetic form of the tetrahydrobiopterin BH_4 co-enzyme, which can stabilize the PAH enzyme in some responsive patients and thus lower phenylalanine levels in blood (50). A compliment to the dietary restrictions is the addition of large neutral amino acids, which reduces phenylalanine concentrations in the brain by competing with phenylalanine for transport across the blood–brain barrier (51).

1.1.9 Maple Syrup Urine Disease

Maple syrup urine disease (MSUD), is yet another example of inborn metabolic diseases associated with the liver and has an incidence of 2 in 100 000 newborn babies in Sweden (Swedish National Board of Health and Welfare). MSUD is caused by a deficiency in the enzyme branched-chain 2-keto acid dehydrogenase (BCKDH) complex involved in the metabolism of branched-chain amino acids (leucine, isoleucine and valine). Loss of function in the BCKDH – less than 2% enzymatic activity – results in elevated plasma levels of branched-chain amino acids. The disease is usually presented in newborns with delayed development, feeding difficulties and the characteristic maple syrup odor in urine, which gives the disease its name. Irreversible neurological damage may ensue. Delayed disease presentation is found in other phenotypes of the disease, such as the intermediate, intermittent, and thiamine-responsive types. The disease occurs globally but is more prevalent in the Old Order Mennonite community of Pennsylvania, USA. Like many other metabolic diseases, the condition can be somewhat stabilized by dietary control or thiamine supplementation in responsive patients but is ultimately subject to liver transplantations which brings a curative treatment (52).

1.1.10 Crigler-Najjar syndrome

Impaired bilirubin metabolism caused by absence or deficiency in the uridine diphosphate glucuronosyltransferase 1A1 (UGT1A1) enzyme leading to bilirubin accumulation and causing Crigler-Najjar diseases type 1 and 2, and type 3 (Gilbert's disease) (53). The disease is rare with an incidence of 1/1 000 000 and was first described 1952 by John Crigler and Victor Najjar (54). Crigler-Najjar patients may develop neurotoxic effect due to unconjugated hyperbilirubinemia and are generally treated with phototherapy to help reduce the bilirubin load. In contrast to type 1 disease, which is a nonexpresser of UGT1A1, type 2 disease retains small residual UGT1A1 expression/activity, which can in turn be induced by phenobarbital. Liver transplantation is a curative option for these patients (55). As for all monogenic diseases, gene therapies may comprise future promising treatment options.

1.2 LIVER TRANSPLANTATION

Pioneered in the 1960s by Thomas Starzl in Denver, CO, USA, liver transplantation (LT) had a rough start with dismal outcome (56). Improvement in organ preservation and immunosuppression ensued with reports of the first successful cases by Starzl, and Roy Calne and Roger Williams from the UK (57, 58). The following decades saw the introduction of improved immunosuppression with ciclosporin and tacrolimus, which dramatically improved long-term survival. Today, the liver transplantation procedure has been standardized, is performed worldwide and is the preferred treatment for many types of end-stage liver disease. Substantial improvements in immunosuppression and advances in the pre- and peri-operative care, in conjunction with advanced multidisciplinary care has reduced mortality and contributed to the success of liver transplantation. The greater success and broader indications for liver transplantation have enabled liver transplantation as a curative treatment for many patients, which creates an unmet need for the available deceased donor organs for transplantation. Living donor programs has been implemented to increase donor livers for transplantation. Limited availability of organs suitable for transplantation remains one of the largest challenges facing the field of transplantation (59)

1.2.1 Domino liver transplantation

Domino liver transplantation (DLT), as schematically shown in (**Figure 2**) is the procedure where the explanted liver from a patient undergoing LT due to one of several metabolic liver diseases (commonly; FAP and MSUD) is used as a donor graft for another patient. DLT has been used to reduce organ shortage and expand the pool of organs that can be used for transplantations (60). The liver from FAP patients are morphologically and functionally intact and remain healthy apart from the production of this amyloid forming variant TTR. Typically, symptoms due to FAP are not seen until the third or fourth decade of life (61). It was therefore expected that explanted livers from FAP patients would expand the donor pool and recipients of FAP livers could be free of symptoms for up to 20 years after transplantation.

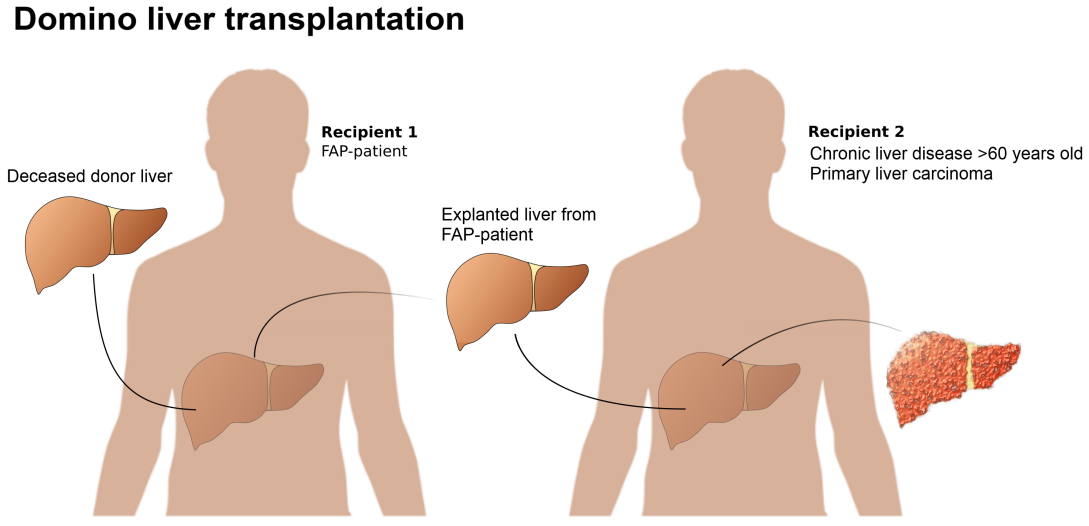

Figure 2. Domino liver transplantation.

Therefore, selected recipients with expected inferior long-term survival and high risk of disease recurrence were likely to benefit from domino liver transplantation. Since 1995 when the first DLT was performed with a FAP donor in Portugal (60), 1254 DLTs have been performed worldwide by December 31, 2017 according to The Domino Liver Transplant registry at the Familial Amyloidotic Polyneuropathy World Transplant Registry (FAPWTR) (38). Similar survival and results to conventional LT have been reported for DLT (62). Mainly patients older than 60 years or patients with hepatocellular carcinoma have been selected for and offered DLT. The impact of this domino-program on patient survival rate and time spent on waiting list has not been evaluated at our center and was addressed in (**Study III**). Shorter waiting times were expected as these domino candidates were waiting on two waiting lists, both the conventional and the domino list.

1.3 MODIFIED MESSENGER RNA THERAPY

Safe, efficient and non-viral mRNA delivery is emerging as a promising therapy for many diseases. The concept is to deliver *in vitro* transcribed mRNA encoding proteins, which ultimately are targets for disease modification or treatment (63). The modified mRNA is produced into mature single stranded mRNA with 5' cap and 3' poly (A)-tail in order to be available for translation by the cellular machinery to generate bioactive proteins (64). This platform offers a temporal aspect that is unique where it is possible to get a pulse-like expression during a limited time frame. Also, modified mRNA has several advantages over gene therapy approaches using virus- and DNA-based vectors, such as avoiding the risk of tumorigenicity, suboptimal genome integration, and ectopic sustained expression. Ease and cost of manufacture are also important aspects to consider in favor of modified mRNA compared to other replacement or gene editing options.

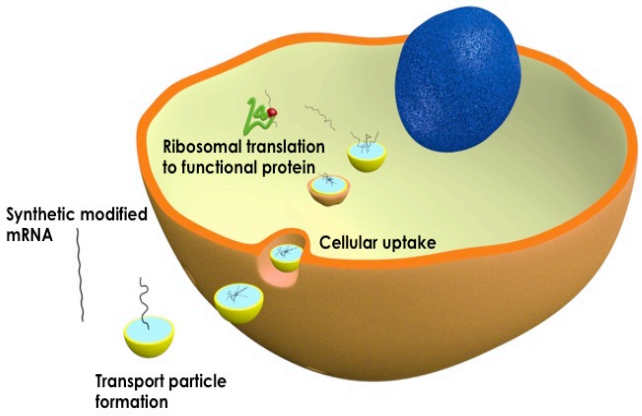

Figure 3. Synthetic mRNA is packaged and transported intracellularly where it is are translated into functioning proteins by the cellular machinery.

Use of nucleic acids as pharmacological compounds have developed substantially since its early proposal using naked RNA and DNA injection into mouse muscle tissue (65). Major challenges to overcome prior to the implementation of RNA as therapy were the fragile nature of RNA and its susceptibility to ubiquitous ribonucleases. Introducing poly A-modifications and using pseudonucleotides addresses this fragility (63, 64, 66). Another major challenge is the immunogenic nature of free RNA molecules that are readily recognized as a molecular pattern associated with viral infections. This pattern is recognized by the intra-cellular toll

like receptors (TLR) of the innate immune system, especially TLR 3, 7 and 8 (67-69). TLR recognition causes inductions of cytokines and gamma interferon (68). Enhanced immunological reaction may be favorable in mRNA-based immunotherapies, it is however an undesirable reaction when considering replacement therapy. Efforts have been made to significantly reduce the immunological footprint of mRNA molecules and it was demonstrated by Karikó et al. that nucleoside modifications with methylcytidine, 2-thiouridine and pseudouridine to the mRNA molecule resulted in reduced dendritic cell activation (70). High purification and modification showed not only reduced immunogenicity of modified mRNA compared to unmodified but also better translational capabilities–further advancing the effort to therapeutic applications of modified mRNA (71, 72).

The next challenge that arises is the intracellular delivery of the mRNA molecule intended for therapy. Initially cationic lipids and recently ionizable lipids are used in the generation of liposomes, lipoplexes or lipid nanoparticles which all facilitate mRNA payload protection, reduced immunogenicity and safe delivery (73-75).

Mechanism for intracellular delivery is shown in (**Figure 3**). The use of modified mRNA delivery is especially enticing for protein replacement therapy. Modification to the synthetic mRNA makes it less immunogenic and incorporation into lipid-based delivery systems eliminates the need for viral particles. Another advantage over DNA-based solutions is the lack of genome manipulation and incorporation, making it safer and enabling the flexibility of transient protein expression.

Areas of therapeutic interest include immune- and replacement therapy. This technology has been used to produce vaccines against the Zika virus and other viruses of pandemic potential (66, 76). Most recently, the same technology is being used in efforts to develop vaccines against the severe acute respiratory syndrome coronavirus 2 and to combat the worldwide pandemic of coronavirus disease 2019 (77).

Use of modified mRNA as a replacement therapy for all genetic/metabolic liver diseases with loss of function is alluring and we studied AATd as a platform in (**Study II**). Trials have been conducted for replacement therapy in methylmalonic acidemia, acute intermittent porphyria, arginase deficiency and also for the expression of factor IX in the treatment of hemophilia A through replacement therapy (78-81).

1.4 HEPATOCYTE TRANSPLANTATION

Being monogenic in nature, gene therapy and gene replacement efforts are particularly suitable and tempting curative options for genetic/metabolic liver diseases. Cell therapies, in particular hepatocyte transplantation are viable options for the treatment of genetic/metabolic liver diseases (82, 83). Enzymatic defects or deficiencies are often manageable with the replacement of only a small percentage of the parenchymal hepatocytes. Hepatocytes are

transplanted through an infusion via the portal vein and is a much less invasive procedure with fewer risks compared to whole organ transplantation (83, 84).

In AATd, studies have shown a repopulation in PiZ transgenic mice after wild-type hepatocyte transplantation (85). It is believed that mutant Z-AAT globules in AATd livers induce a higher cell turnover and may therefore produce a growth-advantage for transplanted wild-type hepatocytes expressing normal M-AAT. Hepatocyte transplantation has also been used in a number of genetic/metabolic liver diseases, including Crigler-Najjar type 1 (83, 86).

Numerous limitations remain for the broader application of hepatocyte transplantation, mainly cell engraftment and retention. Cell source, and maintenance prior to transplantation remain great obstacles, especially the dedifferentiation of hepatocytes in culture and storage. Dedifferentiation is marked by down regulation of phenotypically important proteins paramount for the highly differentiated functions usually available in the observed cell-type (87). Cell-cell interactions and the extra cellular matrix component are all proven to be highly beneficial for the prevention of dedifferentiation (88). These interactions are all achieved to a much higher degree in complex three-dimensional culture systems. A highly studied form of three-dimensional culture is the use of spheroid cultures. Hepatocytes grown as spheroids maintaining complex structures are shown to strongly retain specific hepatic functions and phenotype for longer time-periods surpassing monolayer cultures (89). These complex culture systems may enable multiple cell transplantations and maintenance of a cell source ready for transplantation. Another important aspect of maintaining hepatocytes *in vitro* is the opportunity to provide gene correction and re-transplantation of corrected autologous cells, which was demonstrated in a porcine model of tyrosinemia type 1 (90).

2 AIMS OF THE THESIS

The overall aim of this thesis was to investigate metabolic liver diseases in the setting of liver transplantation, to investigate new experimental treatment options for metabolic diseases and to evaluate the impact of using the livers from metabolic liver disease patients for liver transplantations performed at our center. Studies were performed based on alpha 1-antitrypsin deficiency as an example of common metabolic liver disease and domino liver transplantation procedure with liver from patients with familial amyloid polyneuropathy. Both diseases are hallmarked by primary liver pathogenesis and are both subject to liver transplantation. **Study I** and **II** are pre-clinical and rooted in basic science. **Study III** is based on a retrospective analysis of clinical data.

Specific aims of the thesis:

Study I to examine the hepatic effects of augmentation therapy with pooled plasma purified AAT in AATd patients.

Study II to study the effects of systemic administration of modified mRNA encoding AAT in mice and delivery of modified mRNA to human hepatocytes in culture.

Study III to investigate how the domino liver transplant program impacted patients' waiting time and survival at Karolinska University Hospital.

3 METHODOLOGY

3.1 ETHICAL CONSIDERATIONS

3.1.1 Human samples

The study was approved by the Swedish Ethical Review Authority. Written informed consent was obtained from all patients for participation in the study. All tissue from donor livers had consent for use in clinical transplantation and for use in research. All research was conducted in accordance with the ethical principles of the World Medical Association Declaration of Helsinki (91).

3.1.2 Animal experiments

All animal experiments were conducted in accordance with both European Union, national and local (Karolinska Institutet) guidelines and with approval from The Swedish Board of Agriculture. Animal subjects were kept to minimum numbers sufficient to convey scientific conclusions in each experiment, in accordance with the principles of The Three Rs. C57BL/6J male mice were used in this study. Animals were kept in a temperature-controlled environment with a 12-h/12-h light- dark cycle, with a standard chow and water *ad libitum*.

3.2 PATIENTS AND CLINICAL MATERIAL

Patient data was collected after approval from the Swedish Ethical Review Authority number 2019-04743. Records and waiting time from all LT performed at Karolinska University Hospital, Huddinge, Sweden between 2007 and 2017 were collected from the Nordic Liver Transplant Registry. Additional data on patient characteristics, preoperative illness and status, operative factors, and survival was retrieved from the local registry "Ekvator" and local patient chart software "TakeCare".

Liver tissue used for hepatocyte isolation (**Study I & II**) was acquired with written informed consent from patients undergoing liver resection surgery following primary/secondary tumors or explanted livers from patients being liver transplanted. Also, liver tissue was obtained from donor liver grafts not accepted for transplantation. Tissue collection was conducted after approval from the local ethics committees, the Swedish Ethical Review Authority (Dnr: 2010/678-31/3).

3.3 STUDY DESIGN

3.3.1 Study I

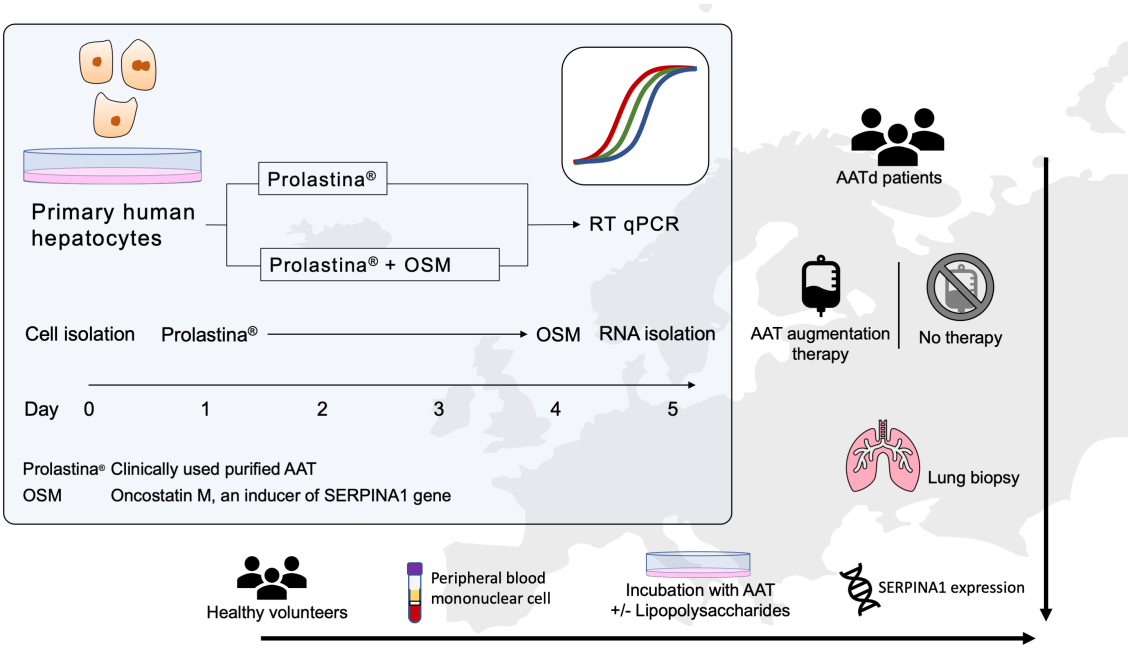

Figure 4. Study design displaying the experimental approach and collaborative aspect of **Study 1**.

Study I was based on the hypothesis that giving augmentation with exogenous AAT protein may reduce the production of mutant Z-AAT protein in AATd patients. This was tested by isolation of primary human hepatocytes from both AATd patient and also from patients with other non-metabolic liver diseases undergoing liver surgery or transplantation (**Figure 4**). Primary hepatocytes were subsequently incubated with purified AAT (Prolastina®, Grifols, Spain), with or without the addition of oncostatin M (OSM), a potent inducer of SERPINA1. After final incubation, RNA was isolated and gene expression of SERPINA1, and other important genes, were quantified.

In parallel, our collaborators in Germany investigated the effect of AAT augmentation therapy on SERPINA1 expression in lung tissue biopsies from AATd patient either receiving augmentation therapy or not. Peripheral blood mononuclear cells (PBMCs) were isolated from healthy volunteers and the cells were incubated with purified AAT in the presence or absence of the stimulant lipopolysaccharides (LPS) to evaluate the effects of AAT on these cells and their SERPINA1 expression. This part of the study was conducted in Germany with collaborators as augmentation therapy is not implemented in Sweden and AATd patient under augmentation therapy are not present.

3.3.2 *Study II*

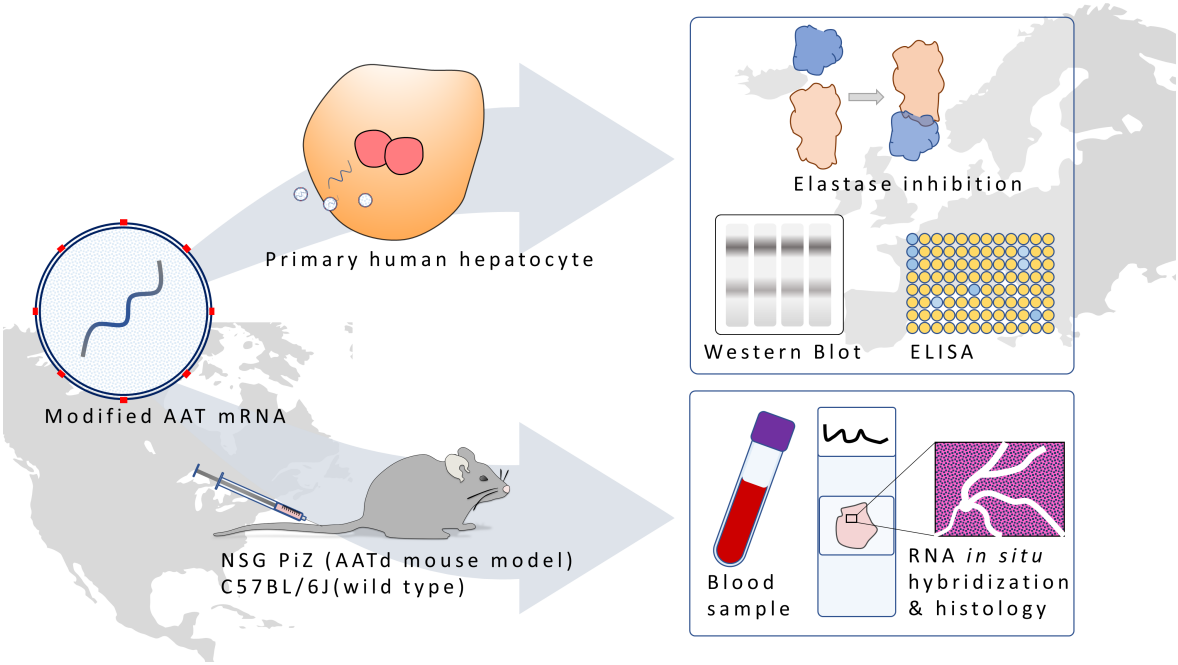

Figure 5. Modified mRNA was tested in primary human hepatocytes from AAT deficient and proficient patients *in vitro*. The mRNA was also delivered *in vivo* in both wild-type mice and the NSG PiZ, AATd mouse model.

In **Study II**, preclinical study, focus was on the molecular aspect of AAT (**Figure 5**). Effort was made towards bringing the promising technology of modified mRNA to AATd patients. Modified mRNA encoding human AAT was manufactured by Moderna Inc (Cambridge, MA, USA). The efficacy of modified mRNA was tested *in vitro* in human primary hepatocyte from AAT-proficient patients and AATd patients. Furthermore, AAT encoding modified mRNA was tested *in vivo* by tail-vein injection into C57BL/6J wild-type mice and in the NOD.Cg-Prkdcscid Il2rgtm1Wjl Tg(SERPINA1*E342K)#Slcw/SzJ, (NSG-PiZ) mouse, a mouse model of AATd. Efficacy of translated AAT-protein following modified mRNA delivery was analyzed by total protein production by Western Blot (WB), secretion (ELISA) and protein activity and function (elastase inhibition). In the animal models, blood samples were collected, and hepatic function and standard liver blood chemistry tests was monitored. Additionally, liver tissue was analyzed via RNA *in situ* hybridization to visualize successful delivery of modified mRNA. Antibody staining of liver tissue was also used to show correct translation and expression of AAT-protein.

3.3.3 *Study III*

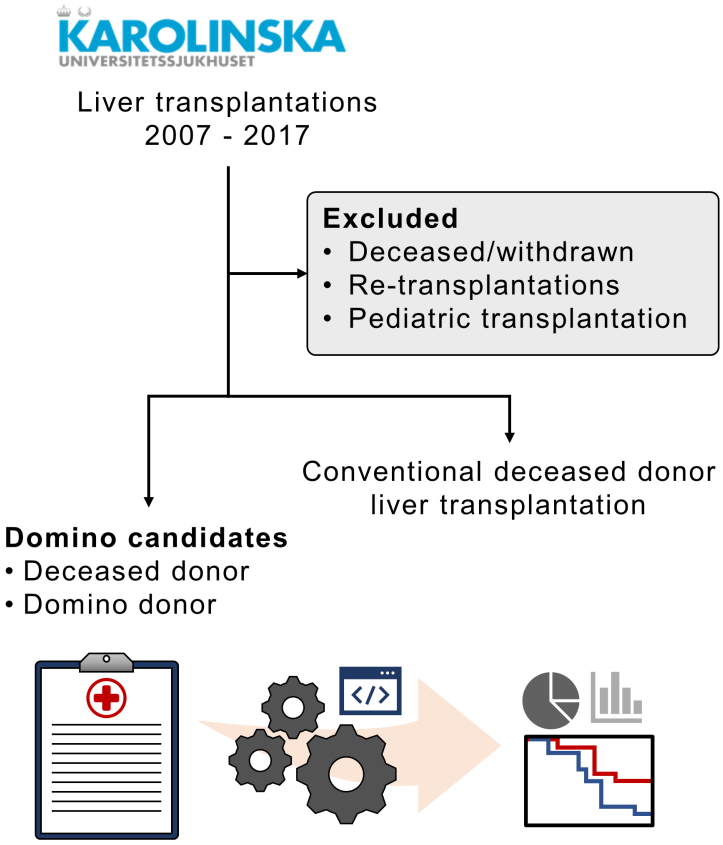

Figure 6. Clinical evaluation of the impact of domino liver program on patient survival and waiting list duration.

Study III, in contrast to the first two studies, is a retrospective clinical study. Its focus lies in the practical challenges and opportunities of metabolic liver diseases in a liver transplantation context. Livers from patients undergoing liver transplantation may in some selected cases and disorders be used as a liver graft for another recipient, enabling the domino liver transplantation procedure. Herein **Study III**, we studied the effect of such program on patient survival and time spent on waiting list for liver transplantation. Data from waiting list and local liver transplant registry was analyzed and all transplantations performed between 2007–2017 at Karolinska University Hospital, Huddinge were screened (**Figure 6**). Patients who died or were withdrawn from the waiting list were excluded. Also, re-transplantation cases were excluded as these re-transplantations differed substantially in rate of both peri- and post-operative complications. Finally, pediatric patients were also excluded. Patients eligible for domino transplantation were identified and divided according to domino outcome, a group that received a domino graft and another that received a deceased donor graft. These two groups were analyzed for both survivals, waiting time and other important pre-, peri-, and post-operative clinical parameters. To juxtaposition these two similar and homogenic groups, they were compared to conventional liver transplantations performed during the same period.

3.4 MOLECULAR BIOLOGY AND LABORATORY TECHNIQUES

3.4.1 Hepatocyte isolation

The human hepatocyte isolation process followed previously established and standardized method of three-step collagenase perfusion technique (83, 92, 93). Major hepatic vessels suitable for cannulation were identified and cannulated with silicone tubing or appropriately sized cannulas. The tissue was continuously perfused via a pump. Firstly, Hank's buffered salt solution (HBSS) supplemented with the chelating agent ethylene glycol tetra acetic acid (EGTA) to remove calcium ions and thus facilitate desmosome disruption was perfused. Secondly, fresh HBSS was perfused to remove all EGTA. Thirdly, a solution of Eagle's minimum essential medium containing collagenase was used to digest the extracellular matrix in the tissue and release the hepatocytes. Liver tissue and solutions were kept at 37° C for optimal enzyme activity and digestion. The tissue was usually digested for 20-30 minutes after which the digested tissue wass manually disrupted and cells were released into suspension. Cold buffer was used to stop the enzymatic activity of the collagenase. Hepatocytes were purified by mesh filtration followed by low-speed centrifugation at 50 g. Cells were resuspended in 4° C William's E medium, with no phenol red. Cell density and viability were determined using trypan blue exclusion method. Isolated primary human hepatocytes were cultured on either collagen coated culture plates or Matrigel, matrix derived from Engelbreth-Holm-Swarm sarcoma (94). Cells were cultured in William's E medium (Sigma-Aldrich, Stockholm, Sweden) supplemented with 20 mM 4-(2-hydro- xyethyl)-1-piperazineethanesulfonic acid (HEPES), 2 mM glutamine, 12 nM insulin, 100 nM dexamethasone, 50 nM amphotericin B and 0.01 M gentamicin. Cells were plated in 12-well plates (Corning Life Sciences, Tewksbury, MA) at a density of 0.75 x10^6 cells per well. The hepatocytes were cultured under standard culture conditions in 37° C incubator with 5% CO_2 and in a humidified atmosphere.

3.4.2 Exogenous AAT treatment

In **Study I**, the effects of exogenous AAT were evaluated. Cells were plated day 0 and divided into one group only treated with purified AAT and the second group co-treated with purified AAT and 10 ng/ml oncostatin M (PeproTech inc. Rocky Hill, NJ, USA) on the final 24 hours of culture. Treatment with purified AAT (Prolastina®; Grifols, Barcelona, Spain) was started day 1 of culture and continued through day 5.

3.4.3 mRNA expression

Analysis of different mRNA expression levels to study the effects of treatments were carried out through **Study I** & **II**. RNA was extracted using the TRIzol® reagent (Invitrogen, Carlsbad, CA). One µg RNA was reverse transcribed using MultScribe™ enzyme (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA). TaqMan® assays specific for (PPIA, SERPINA1, CYP7A1, AFP, MKI67 and PCNA) containing primers and probes were used. Quantitative polymerase chain reaction (PCR) was performed on an ABI Step-One Plus

(Applied Biosystems) instrument. The relative mRNA expression was calculated using PPIA (Cyclophilin A) as an endogenous control gene as described in (95).

3.4.4 SNP analysis and genotyping

Characterization of the different gene variants for the AAT encoding gene SERPINA1 was of high importance. Samples were genotyped for SERPINA1 variants using Single Nucleotide Polymorphism (SNP) genotyping TaqMan® assays on a ABI Step-One Plus (Applied Biosystems) quantitative PCR instrument using the allelic discrimination method according to manufacturer's instruction (96). All probes were pre-designed SNP Genotyping Assay and purchased from Applied Biosystems. For PiS, the rs1758 (assay ID: C_594695_20) probe was used; for PiZ allele, the rs28929474 (assay ID: C_34508510_10) was used; for the PiM2/M4 allele, the rs709932 (assay ID: C_2895146_20) probe was used and finally for the PiNull allele, rs28929473 (assay ID: C_63321235_20) was used.

3.4.5 Phenotyping by isoelectric focusing

Isoelectric focusing was performed on serum samples from individuals with homozygous ZZ genotype using the HYDRAGEL® 18 A1AT IEF Kit (Sebia Inc, Norcross, GA, USA) according to manufacturer's instructions.

3.4.6 Human lung tissue

Lung tissue from surgical resection material from end-stage PiZZ AAT emphysema cases with AAT augmentation therapy (n=10) and without AAT therapy (n=4) were collected. Gene expression analysis was performed as described previously (97-99). Briefly, the samples were cut into 10µm thick sections and suspended in a proteinase K digestion solution overnight. RNA was isolated using phenol-chloroform extraction, followed by ethanol precipitation (97). Complementary DNA was generated and was preamplified with nonrandom PCR primers and PreAmp MasterMix (Applied Biosystems) to lower CT values by 14 PCR cycles (100). Expression of SERPINA1 was analyzed by quantitative PCR.

3.4.7 Human adherent PBMCs

Peripheral blood mononuclear cells were isolated from five healthy volunteers by discontinuous gradient centrifugation, as previously described (101). PBMCs were resuspended in cell culture media and plated at a density of 6×10^6 cells/ml. Monocytes were allowed to adhere to the culture plates, non-adherent cells were washed away. After 24 hours, adherent PBMCs were treated with 1mg/ml AAT (Zemaira, CSL Behring, King of Prussia, PA) alone or together with LPS (1µg/ml) (Sigma) for additional 24 hours.

3.4.8 mRNA transfection

For *in vivo* studies, pre-packaged particles containing mRNA (Moderna Inc., Cambridge, MA) were used and for *in vitro* studies commercial reagents were used. Transfecting agents Lipofectamine™ 2000, 3000, RNAiMax (Thermo Fisher Scientific, Inc., MA) and TransIT®

-mRNA transfection kit (Mirus BIO, LLC, WI) were tested for efficiency and Lipofectamine™ 2000 proved best suited for delivery of modified mRNA in cultured primary human hepatocytes. Modified mRNA was mixed with diluted Lipofectamine™ 2000 at a ratio of 1:3 in Opti-MEM® I (Thermo Fisher Scientific) reduced serum media and incubated at room temperature for 15 minutes for lipoplex formation before being added to hepatocytes. Enhanced green fluorescent protein (eGFP) encoding modified mRNA was used as control of transfection. Liposomes without mRNA content acted as vehicle control, this was selected as minor cytotoxic effect was observed using eGFP as control, which has been previously reported (102, 103).

3.4.9 Protein extraction and immune blotting

To evaluate actual protein translation and production, protein extraction and immune blotting were conducted. Hepatocytes were disrupted and lysed following 48 hours incubation with modified mRNA. Cells were lysed with radioimmunoprecipitation assay (RIPA) buffer (R0278, Sigma-Aldrich, Stockholm) supplemented with protease inhibitor cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail (04693159001**,** Roche Diagnostics GmbH, Mannheim, Germany). Lysates (20 µg protein/lane) were separated by SDS-PAGE (Any kD™ Mini-PROTEAN® TGX™ Precast Protein Gels, 4569033, Bio-Rad Laboratories AB, Stockholm, Sweden) and used for immunoblotting. Anti-SERPINA1 (AAT) (1:2000 dilution, HPA001292, Atlas Antibodies AB, Stockholm, Sweden) and anti-β-actin (1:2000 dilution, ab8227, Abcam plc, Cambridge, UK) antibodies were used. Membranes were developed using WesternBright ECL Kit (K-12045-D20, Advansta Inc, San José, CA) and imaged with Vilber Lourmat UV-instrument (Vilber Lourmat, Collégien, France). Blot images were analysed, and AAT signal was normalized to β-actin signal using ImageJ software (National Institutes of Health, Bethesda, MD) (104).

3.4.10 Enzyme-linked immunosorbent assay

AAT protein concentration in cultured cell media was determined by ELISA 48 hours after transfection. AAT content was analyzed by Human alpha 1 Antitrypsin ELISA Kit (SERPINA1) (ab108799, Abcam plc, Cambridge, UK) according to the manufacturer's instructions.

3.4.11 AAT activity assay

AAT function was determined by EnzCheck Elastase Assay Kit (E-12056, Molecular Probes, Eugene, OR). Briefly, soluble bovine neck ligament elastin labelled with a quenched fluorescent conjugate was used as a substrate. The substrate was digested in the presence of elastin or proteases to yield a fluorescent signal. Working solutions of substrate and provided elastase enzyme from pig pancreas were prepared and combined with cell culture media from vehicle or mRNA treated cells or from serum samples from NSG-PiZ mice. Negative and positive controls were used. Fluorescence intensity was measured every 10 minutes for a total

of 60 minutes using the CLARIOstar microplate reader (BMG LABTECH GmbH, Ortenberg, Germany).

3.4.12 In vivo modified mRNA delivery

First, C57BL/6J mice were dosed with modified mRNA encoding human AAT at 0,5-1 mg/kg body weight in lipid nano particle-formulation as provided by Moderna Inc (Cambridge, MA) via the tail vein. Animals were euthanized 1, 24- and 48-hours post injection. Liver tissue was both snap-frozen and fixed in 10% formalin for immunohistochemistry. Subsequently, male NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl} Tg(SERPINA1*E342K)#Slcw/SzJ, (NSG-PiZ) mice, acquired from The Jackson Laboratories (Bar Harbor, ME) were separated into two groups, 15 animals in the group receiving intravenous mRNA encoding human AAT and 3 animals receiving PBS control injections. The animals were euthanized at 2, 24 and 48 hours after mRNA delivery. Liver tissue was stored by snap freezing in liquid nitrogen and saved in -70°C and by 10%formalin fixation. Blood, lung and intestines were also collected.

Liver associated and general cell damage blood biomarkers aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), creatine kinase (CK), gamma-glutamyl transferase (GGT), and albumin were analyzed in blood samples from all NSG-PiZ animals by IDEXX BioAnalytics (North Grafton, MA).

3.4.13 Immunohistochemistry

Liver tissue was fixed in 4% buffered formalin solution, dehydrated and paraffin embedded. Embedded tissue was sectioned into 5 µm thick sections for further staining. Sections were cooked under pressure for 30 minutes in buffered citrate antigen retrieval solution pH=6 prior to incubation with Background Buster blocking solution (NB306-50, Innovex Biosciences Inc., Richmond, CA) for 20 minutes. Primary rabbit anti-human SERPINA1 antibody (1:200) (HPA001292, Atlas Antibodies) was used and incubated for 60 minutes at room temperature. ImmPRESS® HRP Anti-Rabbit IgG (Peroxidase) Polymer Detection Kit (MP-7451, Vector Laboratories, INC, Burlingame, CA) reagent was used to stain and visualize according to manufacturer's instructions. 3,3'-diaminobenzidine (DAB) staining was compared by measuring reciprocal DAB intensity using ImageJ software according to methods described by Fuhrich et al. (105).

3.4.14 *mRNA in situ hybridization*

mRNA in situ hybridization was performed using RNAscope technology on the Leica BOND RX autostainer platform using the RNAScope® 2.5 LS Reagent Kit-BROWN (Advanced Cell Diagnostics, Newark, CA). An exclusive target probe with proprietary sequences was designed by ACD to target human SERPINA1 mRNA without cross-reactivity to mouse or rat SERPINA1 was used. Control probes to the housekeeping gene Mus musculus peptidylprolyl isomerase B (Ppib), mRNA (cat no 313918, ACD) was used as a positive

control and the bacterial gene dihydrodipicolinate reductase (DapB) (cat no 312038, ACD) as a negative control.

3.5 CLINICAL STUDIES

In **Study III**, the effect of domino liver transplantation on patient survival and time spent on waiting list was evaluated. Pre- and peri-transplant parameters were analyzed as well as post-transplant liver and kidney function. Clinical records and waiting times from all liver transplantations performed at Karolinska University Hospital, Huddinge, Sweden between 2007 and 2017 were collected and analyzed. Waiting times were obtained from the Nordic Liver Transplant Registry. Further, data covering patient characteristics, preoperative illness and status, operative factors, and survival was obtained from patient records "Take Care". Re-transplanted patients were excluded from the study. Survival was calculated from the day of transplantation until November 1, 2019 which was set as the end of the observation point. The study was approved by the Swedish Ethical Review Authority (number 2019-04743) and all procedures were performed in accordance with the guidelines of World Medical Association Declaration of Helsinki (91).

3.6 STATISTICAL ANALYSIS

3.6.1 Study I

Non-parametric Kruskal-Wallis test on ranks with the Bonferroni correction for multiple comparisons was used for multiple group comparisons. Mann-Whitney *U* test or Wilcoxon test (where applicable) were used for comparison between two groups. Test results with p values below 0.05 were considered significant. Analysis was performed in Prism version 6 (Graphpad Software Inc., San Diego, CA).

3.6.2 Study II

Median with interquartile range is presented where non-parametric data is expressed. Individual values are reported, bars show median and interquartile range. Two-tailed tests where p values <0.05 were considered significant. Non-parametric Wilcoxon matched-pairs signed rank test was applied for comparisons between two groups, Kruskal-Wallis test for comparisons was used with the Bonferroni correction for multiple comparisons between more than two groups, as normal distribution could not be assumed. Statistical analysis was performed in Prism version 6 (GraphPad Software Inc., San Diego, CA).

3.6.3 Study III

The Kaplan-Meier estimator with date of transplantation as starting point until either death or end of observation on November 1, 2019 was used to determine survival estimation. Survival between groups was analyzed by the log rank test. Cox proportional hazards model was used to evaluate hazard ratios compared to convLT and for survival and waiting times for the different patient groups (DLT and nonDLT). Operative factors and characteristics relevant to survival and complications were examined for in-between group differences by non-

parametric independent Kruskal-Wallis test with the Bonferroni correction for multiple comparisons. Contingency table and Chi-square test were used to test differences in blood group O distribution across all groups (convLT, DLT and nonDLT).

Data is presented as median, [quartile 1 - quartile 3] in the text and tables. Differences between groups were considered significant in tests with p values <0.05. Statistical analysis was performed in IBM® SPSS® Statistics version 26 (IBM Corporation, Armonk, NY). Figures were made in Prism version 6 (GraphPad Software Inc., San Diego, CA).

4 RESULTS AND DISCUSSION

4.1 **STUDY I** EXOGENOUS AAT

Liver tissue was collected from 15 donors, five donors were AATd patients with either homozygous ZZ-mutations, heterozygous Z- or S-mutations (**Table 1**).

Table 1. Patient samples used in **Study I.**

#	Lab ID	Age	Sex	Diagnosis	SERPINA1 allele
1	HF229	60	F	AATd	ZZ
2	HF312	64	F	AATd	ZZ
3	HF335	18	F	AATd	ZZ
4	HF303	62	M	AATd	Z_
5	VF21	22	M	Organ donor	Z_
6	VF27	61	M	AATd	S_
7	HF238	50	F	CRC metastasis	Not Z
8	HF230	68	M	CRC metastasis	Not Z
9	HF240	36	F	CRC metastasis	Not Z
10	HF235	76	F	Renal cancer	Not Z
11	HF234	59	M	Alcohol cirrhosis	Not Z
12	HF307	35	M	HCC	Not Z
13	HF248	5	F	PFIC2	Not Z
14	VF17	44	M	Organ donor	Not Z nor S
15	VF20	15	M	Crigler-Najjar	Not Z nor S

CRC: colorectal cancer; HCC: hepatocellular cancer;
PFIC2: progressive familial intrahepatic cholestasis type 2

4.1.1 Purified exogenous AAT reduces SERPINA1 gene expression

Focus of **Study I** was the effects of purified AAT treatment specifically on hepatocytes. Augmentation therapy with exogenous AAT is reserved for treatment of AAT-deficiency emphysema. Thus, the possible benefits of this augmentation therapy on the liver is not considered. Treating primary human hepatocytes with purified AAT protein (Prolastina®) reduced the gene expression of SERPINA1 in a dose dependent manner (**Figure 7**). Primary human hepatocytes were cultured and treated with Prolastina®, a commercially available preparation of pooled purified AAT. Gene expression of SERPINA1 (the gene encoding AAT) revealed a decrease in expression when cells were treated with Prolastina® doses of above 7 mg/ml in unstimulated cells. Cultured hepatocytes were stimulated with OSM to accentuate the SERPINA1 expression. Again, the addition of Prolastina® to these OSM stimulated cells showed a decrease of SERPINA1 expression even at lower concentrations (3.5 mg/ml).

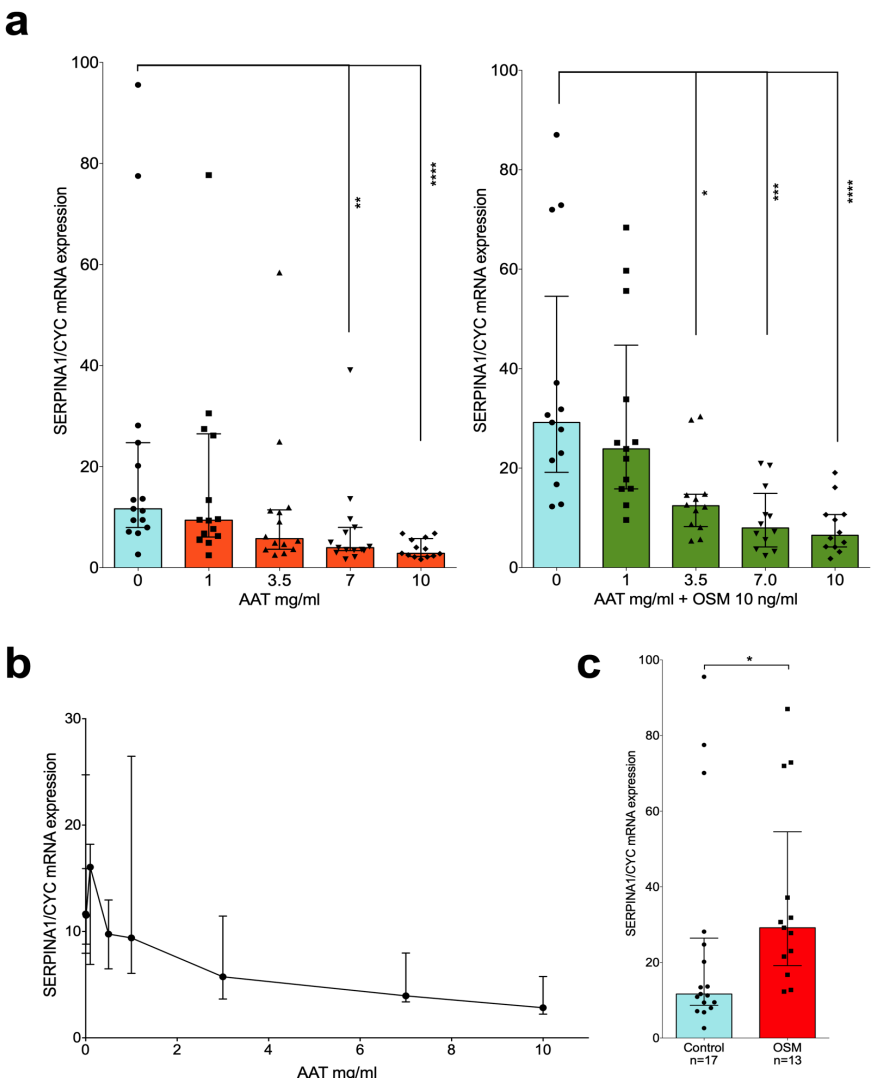

Figure 7. a and **b**, Exogenous purified AAT protein treatment decreases endogenous SERPINA1 gene expression in a dose-dependent manner in primary human hepatocytes. **c**, OSM acts as a potent inducer of SERPINA1. * p<0.05, ** p<0.02, *** p<0.01 ****, p<0.001.

SERPINA1 decrease following exogenous AAT-treatment was further observed in adherent PBMC from healthy AAT-proficient donors. The decrease was importantly observed in lipopolysaccharides-stimulated cells, which had a higher baseline SERPINA1 expression **(Figure 8**). Lung tissue from a limited cohort (n=10) of PiZZ emphysema patients receiving augmentation therapy showed a similar trend with decreased SERPINA1 expression compared to PiZZ patient not receiving augmentation therapy (n=4), (p=0.0539).

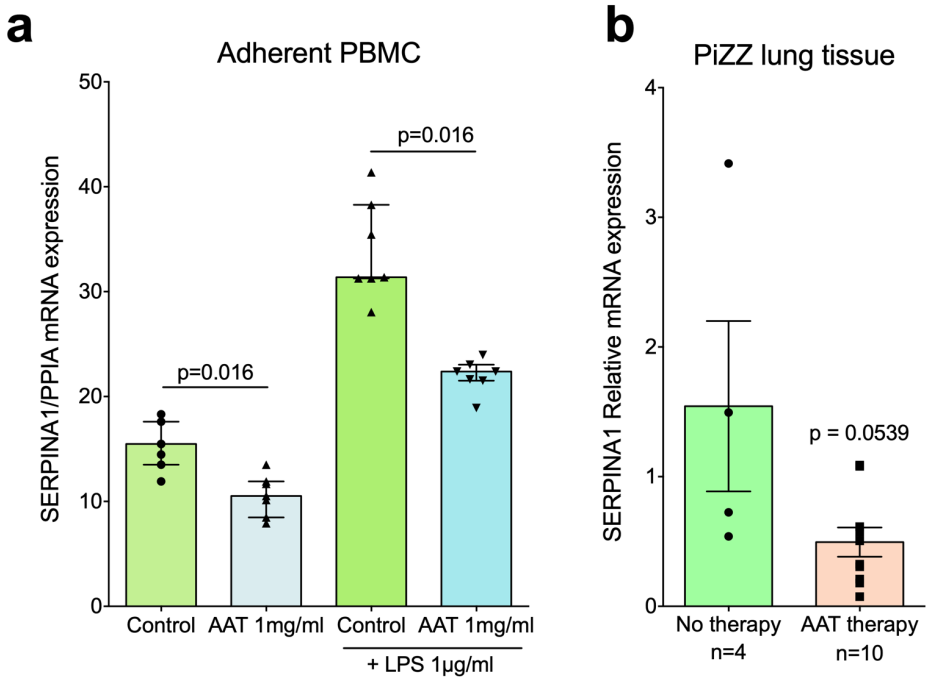

Figure 8. Decreasing SERPINA1 expression in PBMC and PiZZ lung tissue following AAT augmentation therapy.

4.1.2 AAT-deficient and proficient hepatocytes and cellular turnover

Higher cell turnover has been observed in AAT-deficient liver, which led to our further investigation of the cellular turnover in the available material (106). Expression of genes associated with cell proliferation (Ki-67 and PCNA) were investigated in hepatocytes from AAT-deficient and proficient patients and no difference could be found **(Figure 9)**. Higher expression of CASP3, a gene encoding the protein caspase 3, which is associated with apoptosis was present in the deficient cells. Caspase 3 is a zymogen protein staying inactive if not cleaved (107). Although we mainly investigated gene expression by quantitative PCR and did not investigate the protein levels, which is a limitation, these findings may suggest a higher cell turnover and increased apoptosis in the deficient liver. No differences were observed in CYP7A1, AFP nor SERPINA1 expression. AAT-deficient patients showed similar SERPINA1 Expression which further showed the intact production of AAT in these patients and highlighted the cause of deficiency being intracellular entanglement and inability of mutant protein to be secreted into the bloodstream (108).

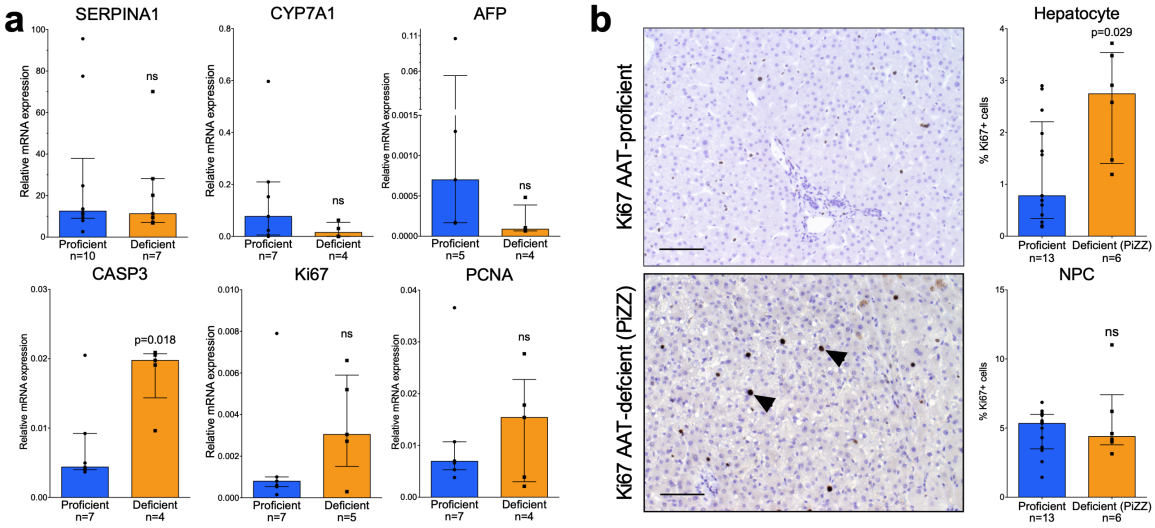

Figure 9. a, Gene expression in hepatocytes from AAT-proficient and deficient patients. **b**, Ki67 stained liver tissue from proficient patients (n=13) and deficient PiZZ (n=6) with subsequent quantification showing increased frequency of Ki67 positive cells (arrow) in the deficient livers. Scalebar denotes 100µm.

Histological evaluation of liver tissue from proficient (n=13) and deficient (n=6) patients further showed an increased frequency of Ki-67 positive proliferating cells in PiZZ deficient livers ($p<0.05$). The Ki-67 positive cells were strictly hepatocytes (**Figure 9**). All deficient livers showed distinct signs of cirrhosis.

4.1.3 Feedback mechanism

Our findings do indeed point to a more complex multifactorial aspect of augmentation therapy with plasma pooled AAT, not strictly limited to pulmonary benefits but a more systemic impact. AAT has been proven having anti-inflammatory and immunomodulatory effects stretching beyond protease activity (109, 110). We hypothesize a possible feedback mechanism in hepatocytes and indeed in other AAT producing cells (out of scope in this study) contributing to AAT homeostasis where increasing levels of circulating AAT may trigger a downregulation of SERPINA1 gene expression (**Figure 10**). Augmentation therapy affected not only hepatocytes but also PBMCs and lung tissue and the conclusions from this study was that expansion beyond emphysema and pulmonary disease should be explored.

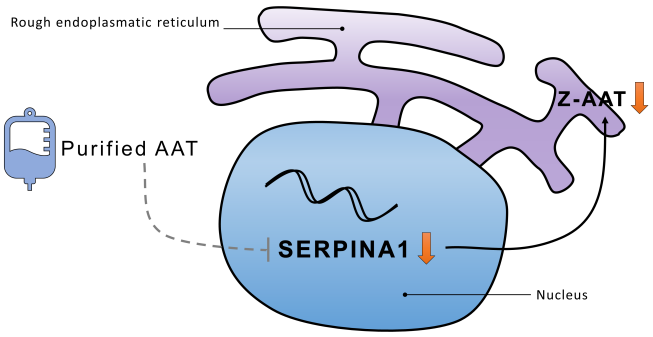

Figure 10. Possible feedback mechanism controlling SERPINA1 gene expression.

4.1.4 Limitations

Ideally, the effects of exogenous AAT on primary hepatocytes should have also been evaluated by measuring secreted AAT protein in media and also quantify the intracellular AAT pool. Such efforts were undertaken but was hindered by the addition of Prolastina®. Prolastina® is purified pooled AAT protein, as such cell incubated with Prolastina® display an intracellular uptake of the protein and Prolastina® is also present in the cell culture media in large quantities. Thus, any efforts to quantify the endogenous AAT protein product was masked by an abundance of added exogenous AAT, i.e. Prolastina®. This fact affected both ELISA and WB assays for AAT as shown (**Figure 11**). A possible workaround, which was considered but ultimately abandoned due to technical issues, was the use of FLAG-tagged modified mRNA encoding AAT. Hepatocytes could be transfected and used to produce FLAG-tagged AAT in culture media. Either filtered or the conditioned media could in turn be incubated with hepatocytes and a possible reduction in endogenous AAT-production could be monitored.

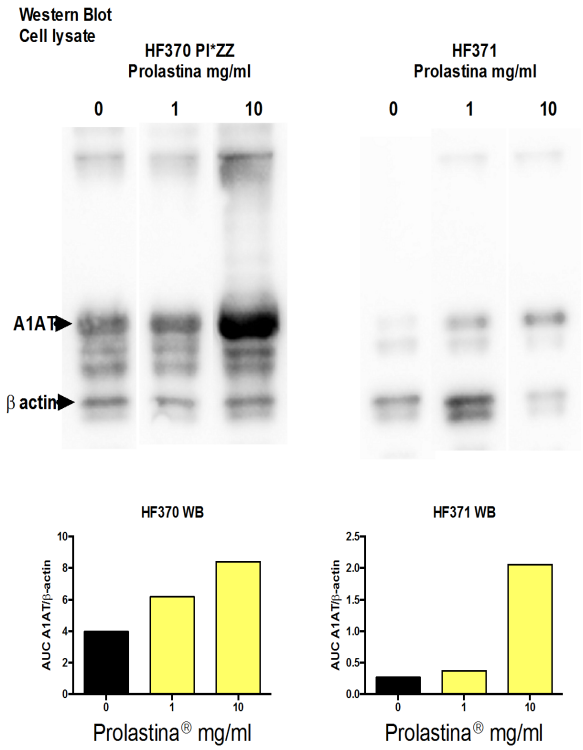

Figure 11. The addition of exogenous AAT, i.e. Prolastina® in large quantity interfered with the interpretation of endogenous AAT and masked any effects as discrimination between exogenous and endogenous AAT could not be performed, given they are identical proteins.

Further, evaluation of SERPINA1 expression in AATd-patients receiving augmentation therapy would be very beneficial in highlighting the possible downregulation of hepatic SERPINA1 expression due to exogenous AAT. This was not possible as AATd patients in Sweden do not receive the option of augmentation therapy. It is ethically unjustifiable to apply potential risks of liver biopsy in AATd patients solely for this study. It may, however, be possible to analyze material from liver biopsies already taken in another study.

4.2 **STUDY II** THERAPY WITH MODIFIED RNA

Modified messenger RNA therapy is an emerging treatment modality displaying promising and encouraging results in many diseases. The modality is particularly suitable for protein replacement as the missing or mutant protein can be corrected by the cellular machinery. In **Study II** we studied the possible use of modified mRNA as a replacement strategy for AAT-deficiency. Codon optimized modified mRNA was produced by Moderna Inc, Cambridge, MA. Preliminary experiments using eGFP was performed to optimize mRNA concentrations and transfection conditions.

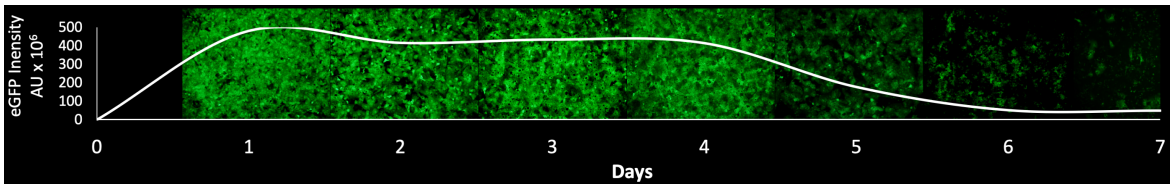

Figure 12. eGFP mRNA expression was used to determine the dynamics of mRNA delivery and translation in primary human hepatocytes *in vitro.*

Primary human hepatocytes were isolated from 16 patients, three of which were explanted livers from AAT-deficient patients undergoing liver transplantation. Hepatocytes were transfected *in vitro* with modified mRNA using lipofection. Hepatocytes from all cases did in fact produce transfected proteins. A rapid production of target protein was seen 2-3 hours after transfection *in vitro*, and production was maintained for up to 5 days (**Figure 12**).

Table 2. Patient samples used in **Study II**.

ID	Diagnosis	Sex	Age (year)	Cell viability %	SERPINA1 Allele
HF370	AATd	M	68	80	Z
HF371	Organ donor	M	6	71	M
HF384	AATd	M	5	74	Z
HF395	FAP	M	31	82	M
HF399	Organ donor	F	40	84	M
HF403	Organ donor	M	68	78	M
HF414	Organ donor	M	27	82	M
HF423	CRC metastasis	M	73	79	M
HF424	CRC metastasis	M	68	67	M
HF425	Cholangiocarcinoma	M	73	71	M
HF426	Unknown tumour	M	71	80	M
HF433	CRC metastasis	M	73	66	M
HF435	CRC metastasis	F	81	66	M
HF440	Organ donor	M	63	80	M
HF455	AATd	M	0.8	78	Z
HF456	Organ donor	M	12	77	M

AATd: alpha 1-antitrypsin deficiency; FAP: Familial amyloidotic polyneuropathy; CRC: colorectal cancer.

After transfection, ELISA analysis of cell culture medium revealed increased production and importantly, secretion of AAT in transfected cells (p=0.0017), which indicates that the produced AAT protein differs from the mutant AAT in that it is secreted and not accumulating in the rough endoplasmic reticulum (**Figure 13**). Also, gel separation and immunoblotting of hepatocyte homogenate showed increased AAT protein production in hepatocytes exposed to modified mRNA. Further analysis of elastase inhibition showed functional activity of the translated protein, which demonstrated inhibition comparable to chemical inhibitors and significant larger inhibition compared to vehicle treated cells (**Figure 14**).

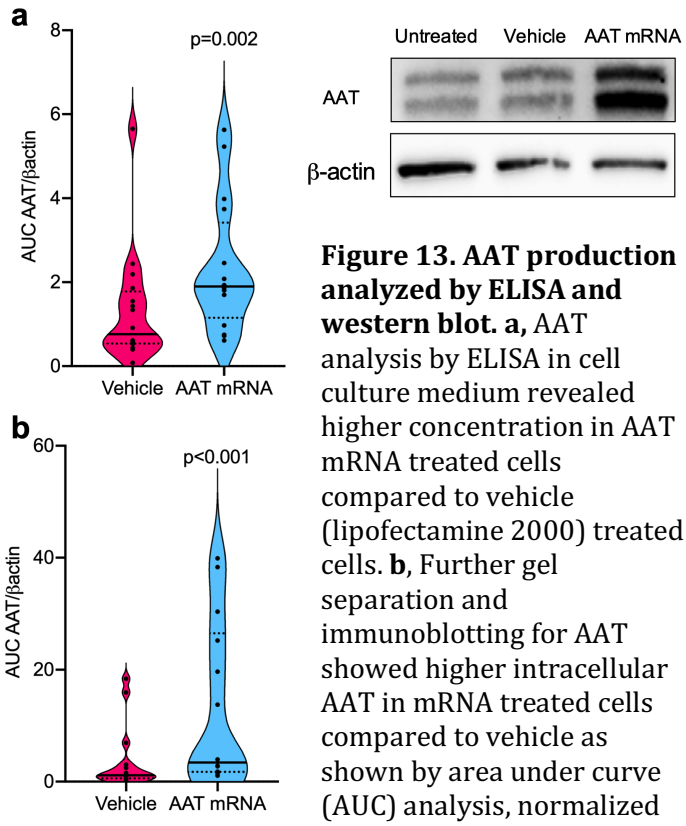

Figure 13. AAT production analyzed by ELISA and western blot. a, AAT analysis by ELISA in cell culture medium revealed higher concentration in AAT mRNA treated cells compared to vehicle (lipofectamine 2000) treated cells. **b**, Further gel separation and immunoblotting for AAT showed higher intracellular AAT in mRNA treated cells compared to vehicle as shown by area under curve (AUC) analysis, normalized

Modified mRNA has been previously used in the treatment of several metabolic liver diseases such as acute intermittent porphyria, methylmalonic acidemia and arginase deficiency (78-80). It has been shown to be successful in replacing or augmenting the disease phenotype and efforts are being taken to introduce mRNA therapy into clinical practice.

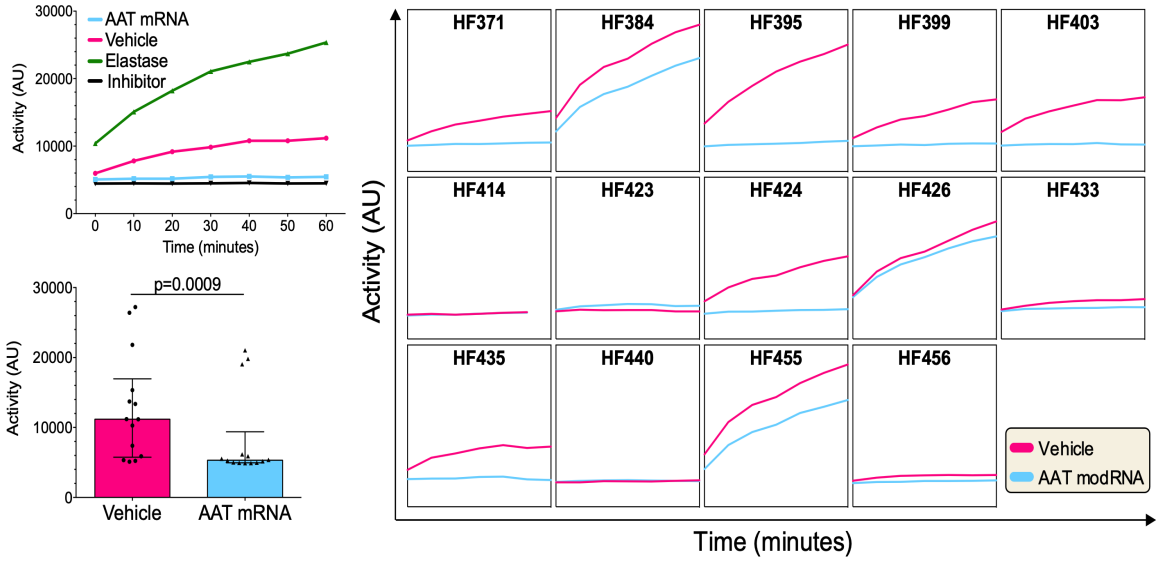

Figure 14. Elastase inhibition/AAT activity assay. All individual cases are represented.

Pulmonary manifestations of AATd has not been replicated in relevant animal models and is therefore difficult to experimentally study and draw out solid conclusions. Liver manifestation of AATd has however been recapitulated in the PiZ transgenic mouse and in the NOD.Cg-Prkdcscid Il2rgtm1Wjl Tg(SERPINA1*E342K)#Slcw/SzJ, (NSG-PiZ) mouse model, used in this study (111, 112). The expression of PiZ AAT in mouse livers have been demonstrated to show Z-AAT globules and accumulation of mutant protein in hepatocytes causing liver fibrosis and HCC (113, 114).

In this study, we transferred the mRNA technology from *in vitro* to *in vivo* to investigate if this methodology would be successful in producing functional, circulating AAT-protein. Modified mRNA was delivered via tail vein injection, first in wild-type C57BL/6J mice and secondly in NSG-PiZ mice. The modified mRNA was packaged in lipid nano particles, which enabled safe delivery. Both minimizing immune responses against foreign mRNA and protecting the mRNA package is paramount to successful *in vivo* delivery and to clinical translation (115, 116).

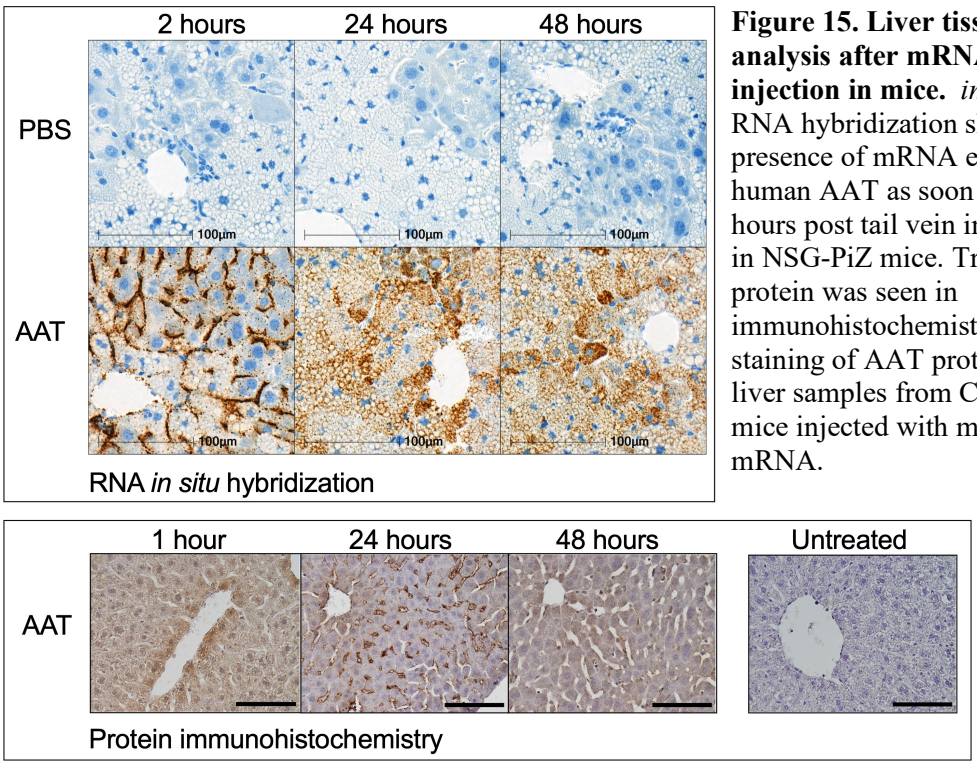

Figure 15. Liver tissue analysis after mRNA injection in mice. *in situ* RNA hybridization showed presence of mRNA encoding human AAT as soon as 2 hours post tail vein injection in NSG-PiZ mice. Translated protein was seen in immunohistochemistry staining of AAT protein in liver samples from C57BL/6J mice injected with modified mRNA.

Modified mRNA-based replacement therapy may also extend treatment to patients with non-severe mutations such as S or heterozygous Z alleles for whom pulmonary deficiency may not be present although these mutations may cause severe liver disease (114). Histological analysis of liver tissue from wild-type mice showed translated AAT protein in hepatocytes as early as 1 hour following tail vein injection and was maintained until end of observation 48 hours after, as shown by anti-SERPINA1 antibody staining for AAT-protein (**Figure 15**). Tail vein injection of modified mRNA in the NSG-PiZ mouse model of AATd was followed up by RNA *in situ* hybridization in liver tissue to visualize the delivery and

cellular uptake of modified mRNA (**Figure 15**). Two hours after injection, human AAT-mRNA could be detected in the liver sinusoids and continued observation showed the cellular uptake throughout hepatocytes. No signs of elevated standard liver blood chemistry tests were detected.

4.2.1 Limitations

Longitudinal studies could have been implemented to investigate the long-term effects of normal M-AAT production on liver disease progression or perhaps even regression. As we previously reported and included in this thesis as **Study I**, normal AAT protein may reduce the production of mutant Z-AAT and thusly a regression of liver disease may be speculated (117). Most likely significant changes in liver morphology and effect on Z-AAT globules would be present after several weeks (months) of continued treatment (24).

4.3 **STUDY III** DOMINO LIVER TRANSPLANTATION

In **Study III**, mainly survival and waiting time duration were evaluated for DLT patients compared to DLT candidates who received deceased donor liver grafts. No major differences were detected between domino graft recipients and candidates intended to be transplanted with a domino liver but were transplanted with a liver graft from a deceased donor. DLT was employed to increase the organ pool and shorten time spent on waiting list.

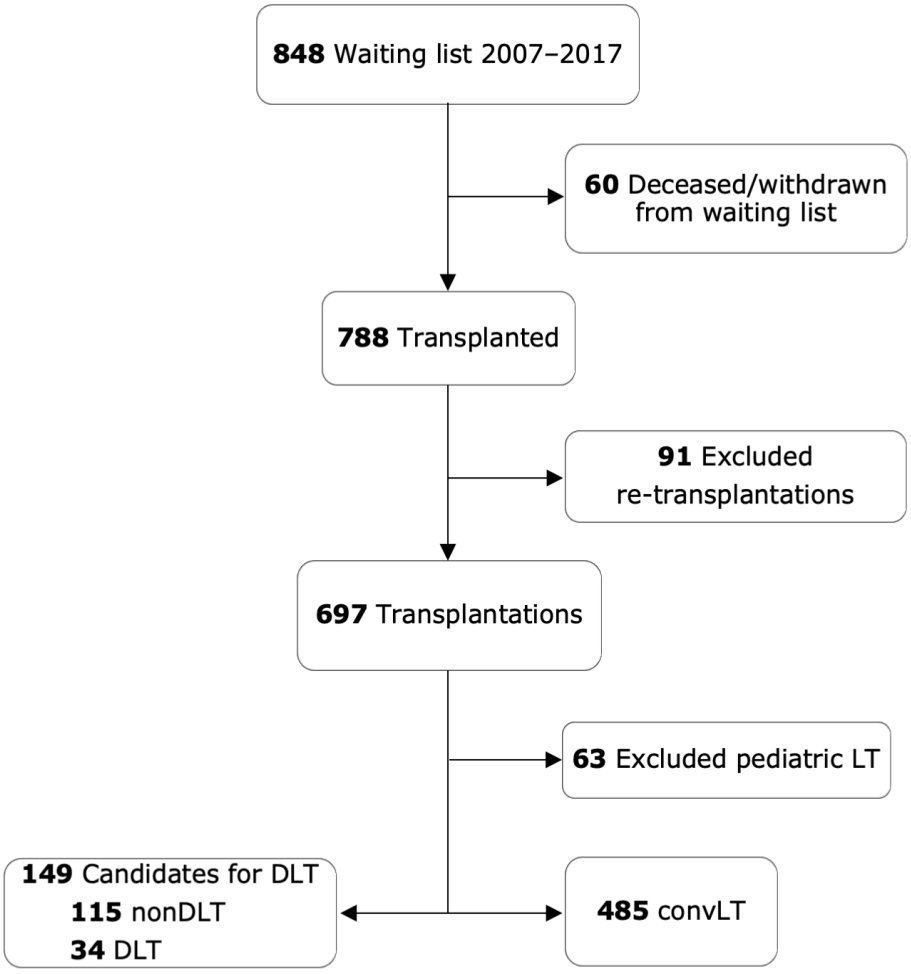

Figure 16. Case selection and transplantation groups.

We identified 634 liver transplantation cases; 485 patients underwent conventional liver transplantation (including 48 FAP patients); 149 patients were offered and accepted DLT of whom 34 patients underwent DLT, while 115 of the domino candidates underwent deceased donor liver transplantation (**Figure 16**). Patients´ sex, age and underlying diagnosis necessitating a liver transplantation is shown in (**Figure 17**).

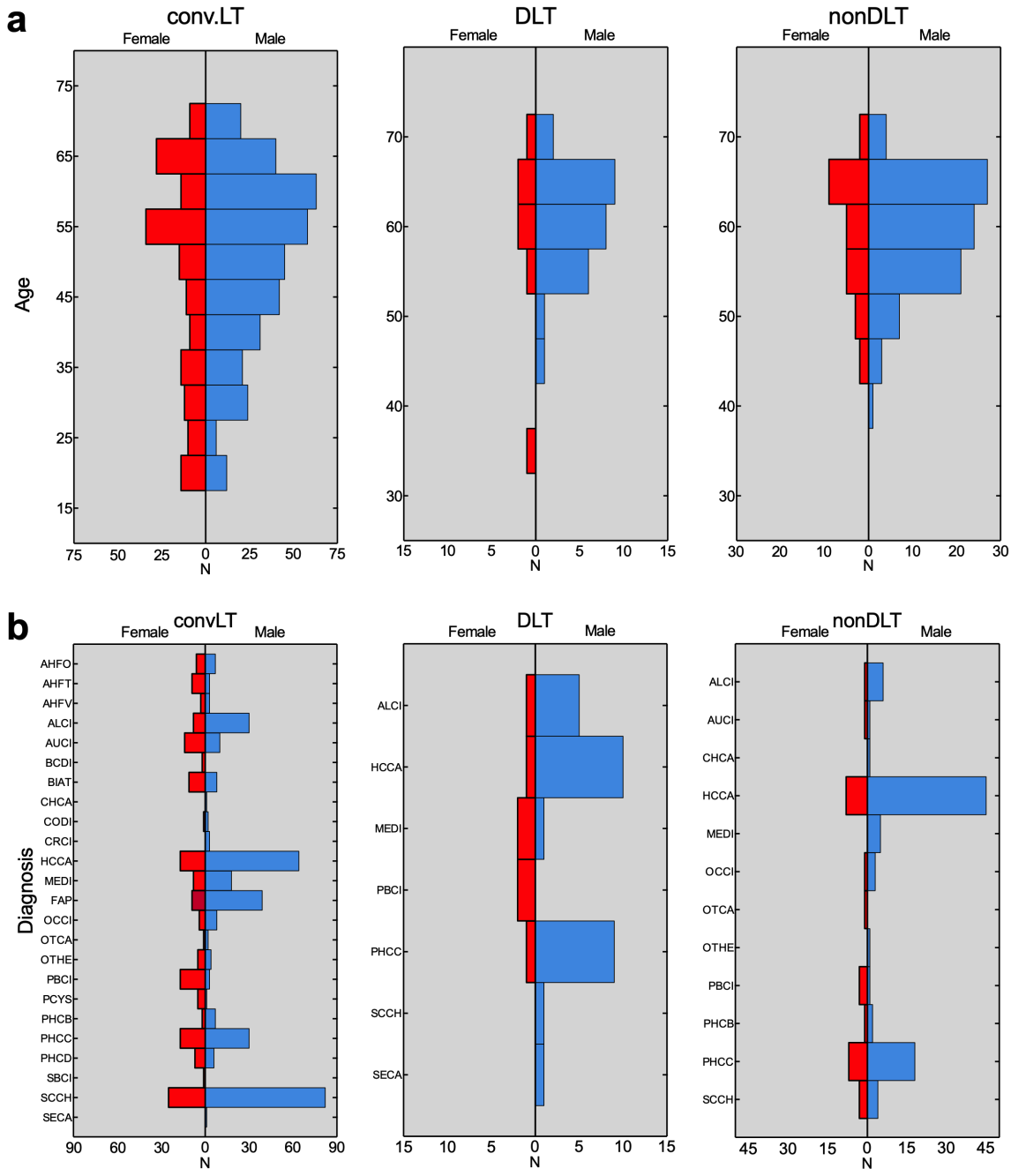

Figure 17. Distribution and characteristics of the different transplant groups.

Five years and overall estimated survival was 84.7% / 60.6% for convLT, 79% / 54.4% for DLT, and 67.6% / 46.7% for nonDLT ($p<0.001$, Log rank). Observed total mortality rates were 43.5% (50/115) for nonDLT and 38% (13/34) for DLT for the total observation time. Median survival was 6.24 years [3.23–8.53] for nonDLT and 6.42 years [3.85–8.82] for DLT.

Cox proportional hazards regression analysis estimated hazard ratio (HR) and 95% Confidence Interval (CI) showed HR 1.72 (95% CI: 0.96–3.06) for DLT and HR 2.02 (95% CI: 1.43–2.84) for nonDLT compared to convLT presented in (**Figure 18**). The Cox regression analysis indicated significant differences in HR for nonDLT compared to convLT but not when comparing DLT to convLT or to nonDLT.

Shorter waiting time for patients accepting a domino procedure were expected since these patients had a chance of undergoing transplantation with both a domino graft and a deceased donor liver. However, our study showed that DLT does not affect the waiting time for LT or survival in patients receiving DLT neither in those who were considered to be candidates for domino transplantation.

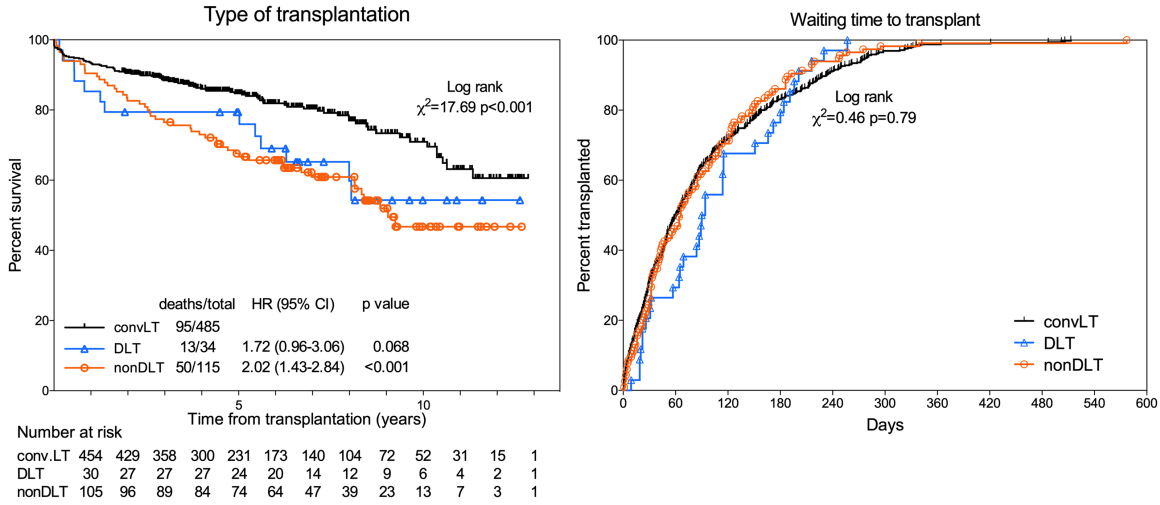

Figure 16. Survival and hazard ratio (mortality), and time spent on waiting list for domino liver transplant patients, non-domino recipients and conventional liver transplant patients.

Alarmingly, symptoms of polyneuropathy and *de novo* amyloidosis in recipients of FAP livers have appeared earlier than previously anticipated (118-122). At our center a proportion of DLT recipients have indeed developed symptoms around 8 years after LT. This may have further reduced the number of domino liver transplantations with FAP donors as currently FAP patients are primarily treated with medication or experimental gene therapy prior to eventual liver transplantation.

In conclusion, DLT has enabled more transplantations without affecting the waiting time and survival rate of patients accepting domino transplantations and standard patients.

4.3.1 Limitations

During the observational period from 2007–2017, changes in the surgical approach and advances in the pre- and peri-operational care may have had a substantial effect on both mortality and morbidity. It is therefore not unlikely that these factors may have made a case to case comparison less equal. Another factor that may have had an impact on patients ultimately selected for DLT or not was the surgeon making the decision. Both considering patients in worse pre-transplant condition and higher urgency for these essentially living donor DLT grafts. On the hand, the decision might have been influenced by the belief that these graft with pre-existing FAP are in fact marginal graft and therefore better suited for a patient in better condition that is able to withstand potential complications.

5 CONCLUDING REMARKS AND FUTURE DIRECTIONS

Specific metabolic liver diseases may be uncommon but collectively they represent a large patient group with considerable morbidity and mortality. Liver transplantation is a curative treatment for these diseases. Whole organ transplantation is however limited by shortage of donor organs for transplantation. Although improved and standardized—liver transplantation *per se*, remains a demanding procedure with many potential complications and life-long immunosuppressive therapy. Therefore, alternative therapies such as enzyme replacement, as suggested herein with modified mRNA are alluring therapies. Certainly, gene modification and therapy could be groundbreaking alternatives.

- Augmentation therapy with exogenous AAT may downregulate the SERPINA1 gene expression and thereby reduce hepatic damage caused by mutant Z-AAT overproduction.

Study I shows a possible unidentified aspect of enzyme replacement and augmentation in AATd, in which the liver protective aspect of enzyme replacement may present an additional beneficial outcome. We speculate that there might be a feedback mechanism downregulating the expression of mutant Z-AAT when adequate circulating AAT is present. Further studies are needed to investigate this possible feedback communication.

- Modified mRNA encoding SERPINA1 (AAT) could be a viable therapy for AAT replacement and augmentation.

In **Study II**, it was possible to both deliver and express modified mRNA encoding AAT. This was possible in both primary human hepatocytes *in vitro* and the NSG-PiZ AATd mouse model *in vivo*. Modified mRNA as a technology is rapidly improving and translational phase II and III studies are underway for use in humans. Naturally, following extensive safety trials, AAT encoding mRNA can be evaluated as a replacement therapy for AATd patients.

- Domino liver transplant has been used to expand the donor pool for whole organ transplantation. The domino program at our center did not affect survival or time spent on waiting list. It did, however, not lengthen the waiting time and may have enabled additional transplantations otherwise not possible considering the mortality on the waiting list.

Whole organ transplantation for patients with metabolic liver diseases has enabled a new source of morphologically healthy and normal organs and created an opportunity to expand the donor pool by domino transplantation. **Study III** shows that patients included in the domino liver transplant have similar survival and waiting time compared to those who underwent conventional liver transplantation. Transfer of *de novo* FAP i.e. the production of mutant TTR protein and amyloid formation in the recipient of a FAP liver has emerged as substantial factor to consider when undergoing domino liver transplantation. The extent of *de*

novo FAP and subsequent treatment options need to be studied further and guidelines should be created.

At our center, the follow-up of DLT patients will continue and guidelines regarding possible treatment options are under development in collaboration with neurologists. All DLT (FAP-donor recipients) at our center have now undergone abdominal fat biopsies to determine the prevalence of *de novo* FAP amyloidosis and perform neurological examinations for early detection of clinical manifestations of FAP. This is an important step in identifying patients who develop *de novo* amyloidosis, regardless of symptoms, as a majority of patients have amyloid presence in fat biopsies but do not display any clinical symptoms of polyneuropathy.

It has now become important to perform fat biopsies in all domino recipients to determine amyloid formation as new drugs are now available. The progression of amyloid driven polyneuropathy may therefore be halted, something that was not previously possible. A challenging factor in interpreting these results are the impact of pre-transplant neuropathy related to alcohol abuse, aging, immunosuppressive drugs, or occurrence of post-transplant diabetes mellitus, which all may lead to symptoms of polyneuropathy.

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